Studies on pharmacological activities of the cauliflower mushroom *Sparassis crispa*

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Takashi Kimura

CONTENTS

General Introduction

Medicinal mushrooms have been used historically in traditional Asian medicine. Scientific and medical research over the past 2–3 decades in Japan, China, and Korea, and more recently in the United States, has shown the potent and unique properties of compounds extracted from mushrooms for the prevention and treatment of cancer and other chronic diseases. Various important pharmaceutical products with proven medicinal applications have been derived from mushrooms [1)].

Many studies have been performed on the antitumor activity of edible mushrooms, particularly the β-1,3-D-glucan component, which is a well-known biological response modifier.[2)] Two antitumor agents for intravenous administration with a β-1,3-D-glucan structure, namely lentinan and schizophyllan, were isolated from *Lentinus edodes* and *Schizophyllum commune*, respectively. However, to date, very few studies have shown the antitumor effects of orally administered β-1,3-D-glucan.

Sparassis crispa, also known as cauliflower mushroom in English and Hanabiratake in Japanese, is an edible mushroom with various medicinal properties. This mushroom species has been cultivated in Japan about 10 years ago. *S. crispa* has a conspicuous cream-white or yellow color and a large cauliflower-like basidiocarp (Figure 1). *S. crispa* is a brown-rot fungus that primarily grows on the stumps of coniferous trees, and it is widely distributed throughout the north temperate zone.[3)] More than 40% of the dried fruit body of *S. crispa* consists of β-1,3-D-glucan.[4)] Ohno et al. showed that β-1,3-D-glucan from *S. crispa* had antitumor activity against the solid form of sarcoma 180 in ICR mice after intraperitoneal administration.[5)]

This study consists of 2 parts: Part I, Determination of the antitumor effects of *S. crispa* and its related components.

Chapter 1 describes the comparison of the antitumor effect of the fruit body of *S. crispa* with that of its mycelia, which showed that the antitumor activity of the fruit body was much stronger than that of its mycelia.

Chapter 2 describes purification of β-1,3-D-glucan from the fruit body of *S. crispa* and determination of the detailed structure by methylation analysis. Furthermore, I have described the anti-angiogenic functions and anti-metastatic effects of *S. crispa* on neoplasm using different animal models after peroral administration.

To my knowledge, no study to date has described the effects of antitumor compounds from *S. crispa* other than β-1,3-D-glucan. Thus, I tried to isolate the antitumor components from *S. crispa* regardless of their degree of water solubility.

The isolation of low molecular weight fraction (containing no β-glucan) from hot water extract of the fruit body of *S. crispa* and examination of the antitumor effect after peroral administration has been described in the Chapter 3.

The isolation and elucidation of the structure of the novel phthalide compounds, hanabiratakelide A–C has been described in Chapter 4. In addition, I have described the biological activity of hanabiratakelides.

The pharmacological effects, except the antitumor effects of *S. crispa* have been described in Part II. I investigated the various pharmacological activities of *S. crispa* using different animal models.

The effects of oral administration of *S. crispa* on allergen-induced production of immunoglobulin E (IgE) and cytokines in murine splenocytes was examined using ovalbumin (OVA)-sensitized BALB/c mice fed with or without *S. crispa*; these results have been described in Chapter 5. In addition, I examined the effects of *S. crispa* on allergen-specific serum IgE levels and symptoms using the murine allergic rhinitis model.

The beneficial effects of *S. crispa* on glycemic responses and plasma levels of adiponectin and insulin in obese mice with type 2 diabetes have been described in Chapter 6.

To evaluate the effects of *S. crispa* on turnover of the stratum corneum and biosynthesis of soluble collagen in the dermis, I used collagen synthetic activity-reduced model rats (RMRs) fed a low-protein diet in Chapter 7.

Figure 1. *Sparassis crispa*

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Part I

Antitumor Effects and Their Related Components of *Sparassis crispa*

Chapter 1 Comparison of the Antitumor Effect of the Fruit Body and the Mycelia of *Sparassis crispa*

INTRODUCTION

More than 40% of the dried *Sparassis crispa* fruit body consists of β-glucan, which is composed of a backbone of β-(1,3) -linked D-glucopyranosyl residues, and has β-D-glucopyranosyl groups joined through *O*-6 and *O*-2 of D-glucose of the backbone[1)]. The fruit body of *S. crispa* has been reported to have many biological activities, including tumor suppression[1-3)], cancer prevention[4)], improvement of natural killer cell activity[3)], anti-angiogenic effects[1)], anti-allergic effects[3,5)], anti-diabetic effects[6)], platelet anti-aggregation activity[7)], HIV-1 reverse transcriptase inhibition[8)], enhancement of hematopoietic responses[9)], and wound-healing effects[10,11)].

However, there are few reports elucidating the effects of cultured *S. crispa* mycelia. To the best of my knowledge, this is the first study investigating the physiological activity of *S. crispa* mycelia. First, a composition analysis of the β-glucan obtained from *S. crispa* mycelia was performed, using a chemical method, in order to identify the putative active component. Furthermore, the antitumor activity of *S. crispa* mycelia administered orally was investigated in comparison to that of fruit body.

MATERIALS and METHODS

Materials

All reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cultivation of S. crispa mycelia

Liquid medium containing 2% glucose, 1% yeast extract, 0.1% polypeptone N, 0.1% soy flour, and 0.1% potassium dihydrogen phosphate was used to grow *S. crispa* mycelia.

After preculture in Erlenmeyer flasks, mycelia were cultivated in a 30-L jar fermenter at 25°C and pH 4 to 5. After 18 days, cultured mycelia were harvested using a finely woven fabric. The mycelia were air-dried and powdered.

Methylation analysis of β-glucan from the mycelia

To determine the sugar linkage in the β-glucan obtained from the mycelia, methylation analysis was carried out as described previously[1)]. β-Glucan from the mycelia was purified enzymatically from *S. crispa* mycelia. Briefly, powdered mycelia was suspended in 0.08 M phosphate buffer (pH 6.0) and treated with thermostable α-amylase (Sigma, St.Louis, MO, U.S.A.) for 30 min in boiling water. Then, subtilisin A (Sigma) treatment (30 min, 60°C, pH 7.5) and amyloglucosidase (Sigma) (30 min, 60°C, pH 4.3) treatment were carried out in succession, folloId by 80%(v/v) ethanol precipitation. The precipitate was resuspended in water, and dialyzed against deionized water. The dialyzed solution was again precipitated with ethanol (final conc. 80%(v/v)) and dried under reduced pressure. β-Glucan from *S. crispa* was methylated using the Hakomori procedure[12)]. The IR spectrum showed no absorption due to a hydroxyl group, and the compound underwent hydrolysis in the presence of 90% formic acid and then with 1N sulfuric acid. The partially methylated sugar thus obtained was converted to alditol acetate[13)] for gas chromatography–mass spectrometry (GC-MS) analysis. GC-MS analysis of partially methylated alditol acetate was conducted using a JMS DX-303 (JOEL LTD., Tokyo, Japan) apparatus equipped with a fused silica capillary column (SPB-5; Supelco, Japan) (0.25 mm × 30 m) programmed at a temperature of 60°C, which was increased to 280°C at a rate of 8°C/min, with a helium (He) flow rate and ionizing potential of 50 mL/min and 70 eV, respectively.

Animals

Four-week-old female ICR mice were purchased from SLC Inc. (Shizuoka, Japan). They were housed in an animal room under the following conditions: temperature, 22 ± 1°C; relative humidity, 55% ± 5%; and artificial lighting from 8:00 to 20:00. Mice were fed standard chow (CRF-1, Oriental Yeast Co., LTD., Tokyo, Japan) and water was supplied *ad*

libitum. The mice were treated according to the ethical guidelines prescribed by the Animal Study Committee of Unitika Ltd. and the "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain" (Notice no. 88, Ministry of the Environment, Government of Japan).

The antitumor effect of fruit body and mycelia

A Sarcoma 180 cell suspension was subcutaneously implanted (2×10^6 viable cells) into the right upper extremity of each mouse after 1 week of acclimatization. After 1 week, a water suspension (0.05 mL) containing the dry powder of the fruit body or mycelia (corresponding to 30 mg/kg) was administered with the help of a stomach tube. Oral administration was performed daily for 15 days. Tumor size was measured twice a week with a caliper, and tumor volume was calculated using the following formula: $\pi a^2 b/6$, where a is the smallest and b is the largest diameter in millimeters[1)]. All values are presented as the means ± SEM. The significance of differences was determined by 2-way analysis of variance (2-way ANOVA). Probabilities of less than 5% ($p < 0.05$) were considered significant. After 5 weeks of implantation, all the animals (10-week-old) were sacrificed. The tumor weights were determined. Percentage of tumor inhibition was calculated using the following formula: $(1 - T/C) \times 100\%$, where T is the average tumor weight in the treated mice and C is that in the control mice. All data were expressed as the means ± SEM. Comparisons among groups were performed using Dunnett's test. Probabilities of less than 10% ($p < 0.10$) were considered marginally significant.

RESULTS

Methylation analysis of β-glucan from mycelia

The final yield of precipitates was 28%; in contrast, that from the fruit body of *S. crispa* was 65%[1)]. The primary structure of the glucan yielded six peaks, and each was identified by its mass spectrum; these are summarized in Table 1. Interestingly, the chemical composition pattern of the β-glucan from the mycelia differed greatly from that of the

β-glucan from the fruit body. That is to say, the β-glucan from the mycelia had less β-(1,3)-linked D-glucopyranosyl residues and a lower degree of branching than that from the fruit body. It is well known that the biological activities of β-glucan, such as antitumor activity, depend on its structure[14]. Therefore, I performed a comparative analysis of the antitumor activity of *S. crispa* mycelia and fruit body.

Table 1. Summary of methylation analysis of β-glucan from *S. crispa* mycelia.

Bond	Feature	Mycelia		Fruit body[1)]	
		Ratio*	Percentage (%)	Ratio*	Percentage (%)
Glc 1→	Non-reducing end	1.00	21.7	1.00	21.9
→3 Glc 1→	β-1,3-bond	2.12	45.9	2.48	54.4
→6 Glc 1→	β-1,6-bond	0.37	8.1		
→2,3 Glc 1→	Branching site			0.37	8.1
→3,6 Glc 1→	Branching site	0.39	8.5	0.71	15.6
→4 Glc 1→	β-1,4-bond	0.38	8.3		
→6 Gal 1→	1,6-galactan	0.35	7.6		
Total		3.88	100.0	4.56	100.0

*Alditol acetate of 2,3,4,6-tetra-o-methyl glucose (Glc 1→) was adjusted to 1.00.

Comparison of the antitumor effect of fruit body and mycelia

As shown in Figure 1, consecutive ingestion of *S. crispa* fruit body powder suppressed tumor growth, while no such activity was observed on ingestion of the mycelial powder. Although no significant differences in tumor size were observed among groups on particular days, 2-way ANOVA indicated that the tumors in the *S. crispa* fruit body group were significantly smaller than in the other groups. Data of tumor weights on week 5 (shown in Table 2) also revealed around 50% tumor inhibition in the *S. crispa* fruit body group; in contrast, the tumor weight in the *S. crispa* mycelia group was almost equal to that in the control group. The tumor weight tended to be decreased in the *S. crispa* fruit body group than the other two groups ($p < 0.10$).

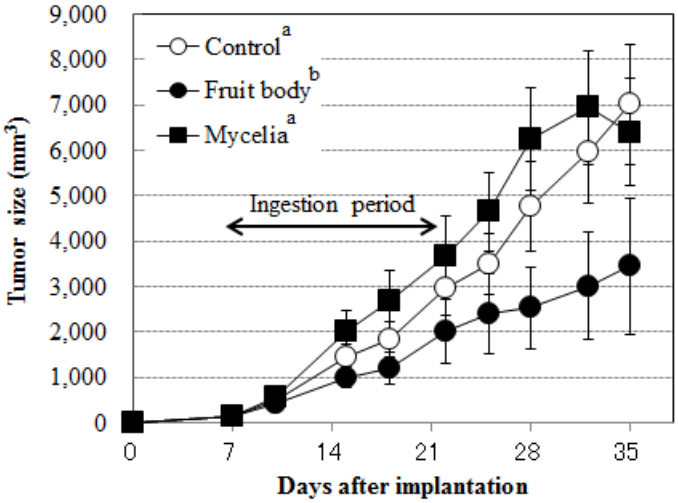

Figure 1. Changes in tumor size of mice orally administered *S. crispa* mycelia or fruit body. The mice were implanted subcutaneously with 2×10^6 Sarcoma 180 cells. A water suspension (0.05 mL) containing the dry powder of *S. crispa* fruit body or mycelia (corresponding to 30 mg/kg) was orally administered daily to the mice implanted with tumor cells for 15 days. Tumor size was measured twice a week with a caliper, and the tumor volume was calculated. Values are means ± SEM of 10 mice per group for each time point. Superscript letters a and b represent significant differences in the mean values ($p < 0.05$, 2-way ANOVA).

Table 2. Antitumor activity of oral administration of *S. crispa* mycelia or fruit body against Sarcoma 180 cells.

Group	Tumor weight (g)	Inhibition percentage (%)
Control	6.0 ± 1.1^a	-
Mycelia	5.7 ± 1.1^a	5
Fruit body	2.8 ± 1.2^b	53

The mice were implanted subcutaneously with 2×10^6 tumor cells. A water suspension (0.05 mL) containing the dry powder of *S. crispa* fruit body or mycelia (corresponding to 30 mg/kg) was orally administered daily to the mice implanted with tumor cells for 15 days. After 5 weeks of implantation, all the animals (10-weeks-old) were sacrificed and tumor weights were determined. Each value is the mean ± SEM of 10 mice per group. Superscript

letters a and b represent marginally significant differences in the mean values ($p < 0.1$).

DISCUSSION

Mushrooms are recognized not only as foods but also as medicines because of their bioactive compounds offering huge beneficial impacts on human health. One of those potent bioactive compounds is β-glucan, comprising a backbone of glucose residues linked by β-1,3-glycosidic bonds with attached β-1,6-branch points, which exhibits antitumor and immunostimulatory properties. In the past, two antitumor agents that are types of β-glucan, namely lentinan and schizophyllan, were isolated from *Lentinus edodes* and *Schizophyllum commune*, respectively. Miyazaki *et al*.[15] suggested that the optimal branching frequency is from 0.2 (one in five backbone residues) to 0.33 (one in three backbone residues). In fact, lentinan (2/5) is a β-1,3-glucan possessing two branches for every five D-glucopyranosyl residues[14]. Schizophyllan (1/3) is also a β-1,3-glucan having one branch for every three D-glucopyranosyl residues[14]. On the other hand, I have already demonstrated that the major structural units of β-glucan from the fruit body of *S. crispa* were a β-1,3-glucan backbone with three side branching units every ten residues (3/10)[1]. In other words, these β-glucans have a very similar structure. However, β-glucan obtained from *S. crispa* mycelia had an obviously lower degree of branching than the above-mentioned optimal branching frequency (below 0.1).

The above-mentioned results suggest that the intergroup difference in antitumor activity might be attributable to the structure and content of β-glucan, which is the putative active component, in the mycelia and the fruit body. Although it has been suggested that dietary *S. crispa* fruit body powder is useful for cancer immunotherapy in combination with lymphocyte transplantation[16], these results might not be applicable for *S. crispa* mycelial powder.

ABSTRACT

To my knowledge, this is the first study in which the antitumor effect of the mycelia of *Sparassis crispa* was investigated. The antitumor effects of the mycelia and the fruit body were evaluated following oral administration at a dose of 30 mg/kg/day to tumor-bearing ICR mice for 15 days. Tumor size was measured for 4 weeks, whilst tumor weight was determined at week 5. The consecutive ingestion of *S. crispa* fruit body powder significantly suppressed tumor growth, while no such activity was observed on ingestion of the mycelial powder. The percentage of tumor weight inhibition was about 50% in the fruit body group, while a slight suppressive effect was observed in the mycelial group. The results of methylation analysis suggested that the structure of the β-glucan obtained from the mycelia differed markedly from that of the β-glucan obtained from the fruit body. The intergroup difference in antitumor activity might be attributable to the structure and content of β-glucan, which is the putative active component.

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Chapter 2 Anti-angiogenic and Anti-metastatic Effects of β-1,3-D-Glucan Purified from *Sparassis crispa*

INTRODUCTION

Sparassis crispa, Hanabiratake in Japanese, is an edible mushroom with medicinal properties, which has recently become cultivable in Japan. Hasegawa *et al.* have already demonstrated the antitumor and antiallergic activities of this mushroom.[1)] *S. crispa* contains more than 40% of β-D-glucan and it has been reported that its major structural unit is β-(1,3)-D-glucan backbone with single β-(1,6)-D-glucosyl side branching units every three residues.[1–3)] However, a detailed structure study of β-D-glucan from *S. crispa* has not been achieved yet by chemical analysis.

β-1,3-D-glucan is a well-documented biological response modifier,[4)] and β-D-glucan extracted from *S. crispa* (SBG) has been reported to possess many biological activities, such as antitumor effects,[1,2)] antiallergic effects,[1)] improvement of natural killer (NK) cell activity,[1)] cytokine-inducing activities in the splenocytes of the mice and human peripheral blood mononuclear cells (PBMC)[5–7)] and enhancement of hematopoietic responses.[(2,8–10)] Furthermore, a recent study has suggested the possibility that the application of SBG in cancer patients could be an effective treatment strategy.[11)] However, besides immunoenhancing activities, the mechanisms of antitumor activities of β-1,3-D-glucan derived from SC have not been investigated thus far.

Angiogenesis is involved not only in the physiological processes such as embryonic development, ovulation and wound healing, but also in many pathological conditions such as solid tumor growth, diabetical retinopathy, age-related maculopathy and rheumatoid arthritis.[12–15)] Vascular endothelial growth factor (VEGF) is the most prominent angiogenic protein often secreted by the solid tumor cells especially under hypoxic conditions.[16–18)] Newly formed blood vessels help in tumor progression and promote metastatic spread of the tumor cells. Therefore, anti-angiogenic strategies are considered to be highly effective in cancer therapy.[16–18)]

In this chapter, I elucidated the primary structure of SBG using methylation analysis, investigated the anti-angiogenic effects of SBG by using two different animal models, and further assessed the anti-metastatic activities *in vivo*. I further analyzed the possible mechanisms of SBG function by using human umbilical vein endothelial cells (HUVECs) *in vitro*.

MATERIALS and METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) was obtained from Nacalai Tesque (Kyoto, Japan). Humedia-EG2 medium was purchased from Kurabo (Osaka, Japan). Antibiotic-antimycotic solutions (100×) containing 10,000 units/ml penicillin, 10 mg/ml streptomycin and 25 μg/ml amphotericin B in phosphate-buffered saline (PBS) was purchased from Wako (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Gibco BRL (Auckland, New Zealand). Diffusion chamber ring, MF cement and 13 mm circular membrane filters were obtained from Millipore (Tokyo, Japan). Growth factor-reduced phenol red-free Matrigel was obtained form Becton Dickinson & Co. (Franklin Lakes, NJ, U.S.A.). Recombinant mouse VEGF was purchased from Sigma (St. Louis, MO, U.S.A.).

Cells

The highly metastatic B16-F10 and B16-BL6 cells were maintained in DMEM supplemented with 10%(v/v) FBS, 1 × antibiotic-antimycotic. HUVECs were obtained from Kurabo and cultured in HuMedia-EG2 medium supplemented with 2%(v/v) FBS, 10 ng/ml human epidermal growth factor, 1 μg/ml hydrocortisone, 5 ng/ml human basic fibroblast growth factor, 10 μg/ml heparin, 50 μg/ml gentamicin and 50 ng/ml amphotericin B.

Animals

Female ICR (4 weeks old) and C57BL/6J mice (5 week old) were obtained from Japan SLC (Shizuoka, Japan), and Clea Japan (Osaka, Japan), respectively. They were housed

for 1 week in a room maintained at 24 ± 1°C with a 12 h light-dark cycle with free access to diet (Labo MR stock, Nihon Nosan Kogyo) and water. The mice were treated according to the ethical guidelines prescribed by the Animal Study of Unitika Ltd.

Purification of β-glucan from S. crispa (SBG)

β-Glucan purified from *S. crispa* was named SBG. SBG was purified enzymatically from the fruiting bodies of *S. crispa* cultivated by Unitika Ltd. Briefly, powdered *S. crispa* was suspended in 0.08 M phosphate buffer (pH 6.0) and treated with thermostable α-amylase (Sigma) for 30 min in boiling water. Then, subtilisin A (Sigma) treatment (30 min, 60°C, pH 7.5) and amyloglucosidase (Sigma) (30 min, 60°C, pH 4.3) treatment was carried out in succession, followed by 80%(v/v) ethanol precipitation. The precipitate was resuspended in water, and dialyzed against deionized water. Inner solution was again precipitated with ethanol (final conc. 80%(v/v)) and dried under reduced pressure. The final yield of precipitates was 65.0%, and the total sugar content was measured as 98.2% using the phenol-sulfuric acid method with glucose as the standard.[19)]

Metylation analysis of SBG

SBG (2.7 mg) was methylated by Hakomori procedure.[20)] The product was confirmed to show no absorption for the hydroxyl group in its IR spectrum, and it hydrolyzed with 90% formic acid and then with 1 N sulfuric acid. The partially methylated sugar thus obtained was converted to the alditol acetate[21)] for GC-MS analyses. Gas chromatograph – mass spectrometer was conducted with a JMS DX-303 apparatus equipped with a fused silica capillary column SPB-5 (Supelco, Japan) (0.25 mm×30 m) programmed from 60℃ to 280℃ at 8℃/min with a gas flow rate of 50 mL of He per min for partially methylated alditol acetate, and the spectra were recorded at an ionizing potential of 70 eV.

Tumor-induced angiogenesis in the dorsal air sac (DAS) assay

DAS assay was carried out according to the method described by Oikawa T., *et al.*[22)] with slight modification. Briefly, both sides of the diffusion chamber ring were covered with

membrane filters, and the resulting chambers were filled with B16-F10 cells (2×10^6 cells) in 150 μl of PBS. Each B16-F10 containing chamber was implanted into the dorsal air sac of female ICR mice on day 0. The negative control group was implanted with PBS containing chamber. SBG was orally administered once a day from six days before to six days after the day 0 for a total of thirteen days. On day 7, each mouse was sacrificed and tumor cell-induced angiogenesis at the implanted zone was observed. The number of newly formed blood vessels > 3 mm in length with a characteristic zigzag shape was counted.

VEGF- induced angiogenesis in the Matrigel plug system

The Matrigel plug assay was performed according to the method described by Kimura Y., *et al*.[23] Briefly, each female C57BL/6J mice was subcutaneously injected with 0.5 ml of Matrigel containing 20 ng/ml VEGF and 32 U/ml heparin on day 0. Negative control group was injected with Matrigel alone. SBG was orally administered once a day from seven days before to five days after the day 0. On day 6, each Matrigel was excised and weighed, and then the gel was treated with dispase-II (1.5 mg/ml, Roche Diagnostics, Tokyo, Japan) followed by determination of hemoglobin content using the Quantichrom hemoglobin assay kit (Funakoshi; Tokyo, Japan).

Tumor growth and spontaneous lung metastasis

Spontaneous pulmonary metastasis assay was carried out based on the method described by Murata J., *et al*.[24] Briefly, highly-metastatic B16-BL6 cells (1.5×10^5) in PBS were injected into the right footpad of female C57BL/6J mice on day 0. SBG was orally administered six times per week from seven days before to forty four days after the day 0. Tumor growth was measured every 3–4 days from day 11 to day 24 and indicated as volume, which was calculated using the following formula: tumor volume (mm^3) = $\pi \times$ (large diameter) × (small diameter)^2/6. To examine lung metastasis, primary tumors were excised on day 24 and the mice were kept alive for another 3 weeks. On day 45, lungs were removed and the number and size of pulmonary metastatic colonies were counted using a stereomicroscope.

In vitro proliferation, migration and capillary morphogenesis of HUVECs

To assess the proliferation of HUVECs, the cells were seeded into a 96-multiwell culture plate at 5×10^3 cells/ml and SBG was added. Proliferation of the HUVECs was measured using the cell counting kit (CCK-8; Dojindo laboratories, Kumamoto, Japan) at 24 and 68 h post seeding. Migration of the HUVEC was examined by wound healing assay.[25] The HUVECs were cultured to confluency in a 24-multiwell culture plate. The monolayer was scratched using 200 μl pipet tips, following which the width of the wound was measured after 9 hours. Capillary morphogenesis was observed using two-dimensional Matrigel-based assay, as described by Stanley G., *et al*.[24] Briefly, 0.2 ml of Matrigel was coated onto a 24-multiwell plate, The HUVECs were seeded at a density of 4×10^4 cells/well, followed by the addition of SBG. Sixteen hours post seeding, capillary morphogenesis was observed using a microscope. Quantification of the capillary tube formation was performed by counting the number of tubule junctions.

Statistics analysis

All data were represented as mean ± SE. Differences among means were analyzed by the Student's *t*-test or Mann-Whitney *U*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Primary structure of SBG

Gas chromatography gave four peaks, and each was identified by its mass spectrum. These are summarized in Table 1. These results suggested that the major structural units of SBG are a β- (1,3)-D-glucan backbone with two β-(1,6)-D-glucosyl side and single β-(1,2)-D-glucosyl side branching units every ten residues, as shown in Figure 1.

Table 1. Summary of methylation analysis of SBG.

Bond	Feature	Ratio*	Ratio (%)
Glc 1→	Non-reducing end	1.00	21.9
→3 Glc 1→	β-1,3-Bond	2.48	54.4
→2,3 Glc 1→	Branching site	0.37	8.4
→3,6 Glc 1→	Branching site	0.71	15.6
total		4.56	100.0

* Alditol acetate of 2,3,4,6-tetra-*O*-methyl glucose (Glc 1→) was adjusted to 1.00.

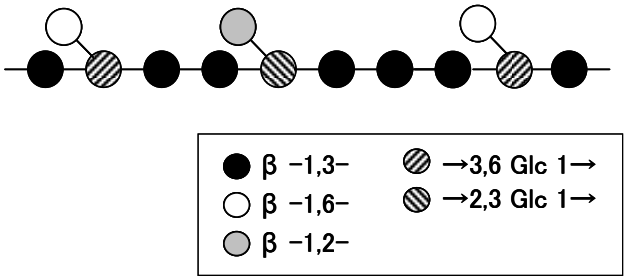

Figure 1. Structure model of SBG presumed by methylation analysis.

Effects of oral administration of SBG on tumor induced angiogenesis

I examined the effects of SBG on tumor-induced neovascularization using the DAS system. Angiogenesis was strongly induced after implantation of B16-F10 cells in the chamber; however, oral administration of SBG significantly inhibited the tumor-induced angiogenesis (Figure 2).

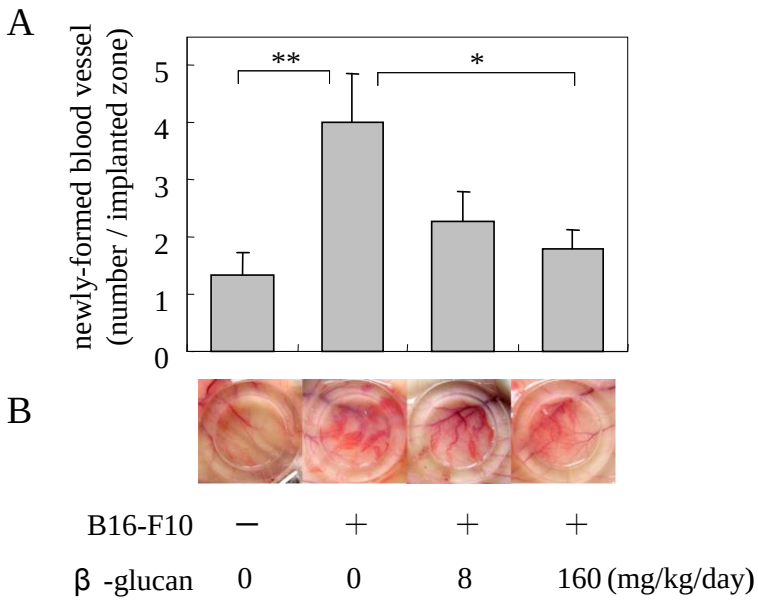

Figure 2. Effects of SBG on tumor-induced angiogenesis in the DAS assay system. The number of newly formed blood vessels > 3 mm length with a characteristic zigzag shape in the chamber implanted zone was indicated (A). Representative photographs at the chamber-implanted zone of each group (B). *: $P < 0.05$, **: $P < 0.01$. N = 6–9.

Effects of oral administration of SBG on VEGF-induced angiogenesis

I investigated the anti-angiogenic effects of β-glucan using another *in vivo* model of angiogenesis, the Matrigel plug assay. Remarkable increase in the Matrigel weight and the hemoglobin content was observed in the group injected with Matrigel containing VEGF compared to the group treated with Matrigel alone. Conversely, such angiogenic responses were significantly suppressed by oral administration of SBG (Figure 3).

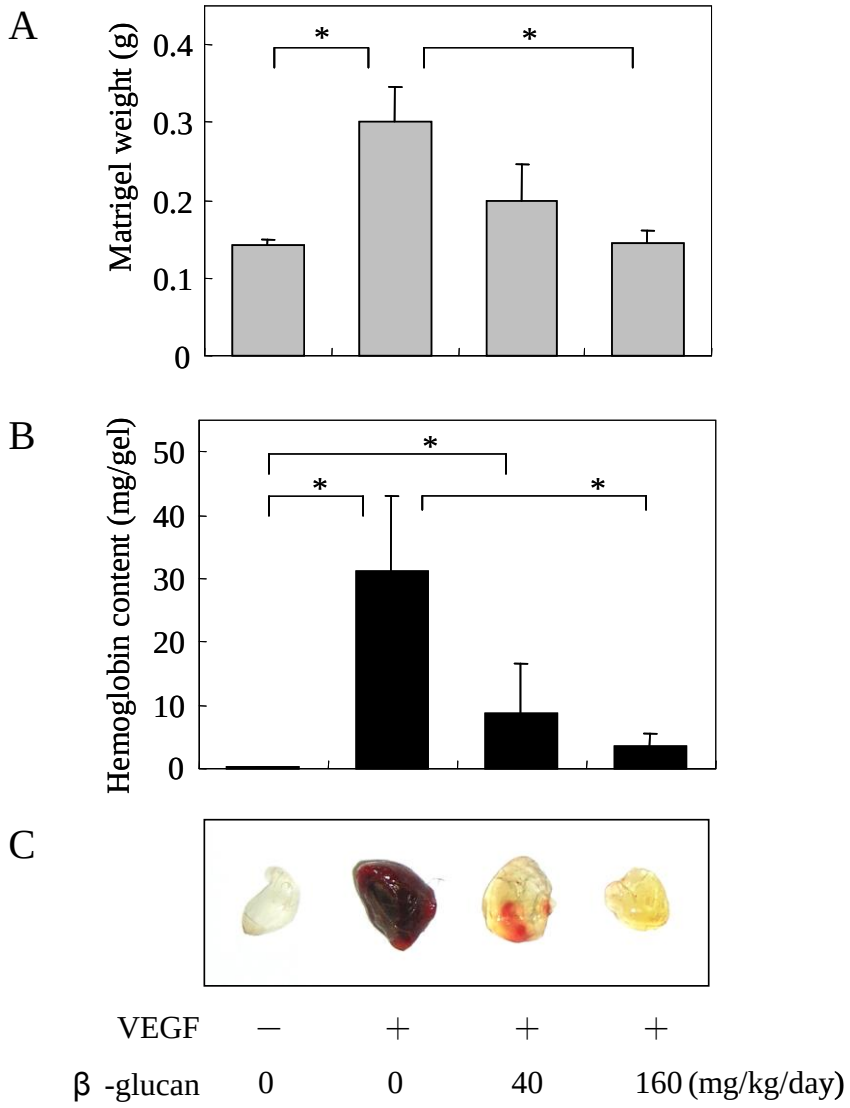

Figure 3. Effects of SBG on vascular endothelial growth factor (VEGF)-induced angiogenesis in Matrigel plug assay. Matrigel weight (A), Hemoglobin content in the Matrigel (B), Representative photographs of excised Matrigels of each group (C). *: $P < 0.05$. N = 5–6.

Effects of oral administration of SBG on tumor growth and lung metastasis

Anti-angiogenic therapy is well-accepted in recent cancer therapy since angiogenesis is an essential event involved in tumor progression and metastasis. I investigated the effect of SBG on tumor growth and lung metastasis. Tumor growth rate of subcutaneously injected B16-BL6 cells was retarded by the oral administration of SBG (Figure 4). Furthermore, the number as well as the size of the pulmonary metastatic tumor colonies was significantly

reduced by SBG administration (Figure 5).

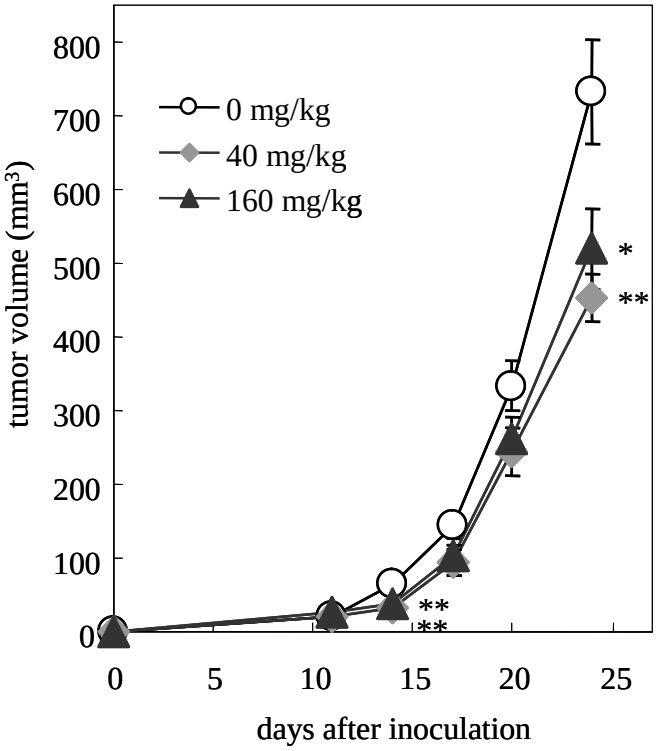

Figure 4. Effects of SBG on primary tumor progression. *: $P < 0.05$, **: $P < 0.01$ (vs control group at each time point). N = 8–10.

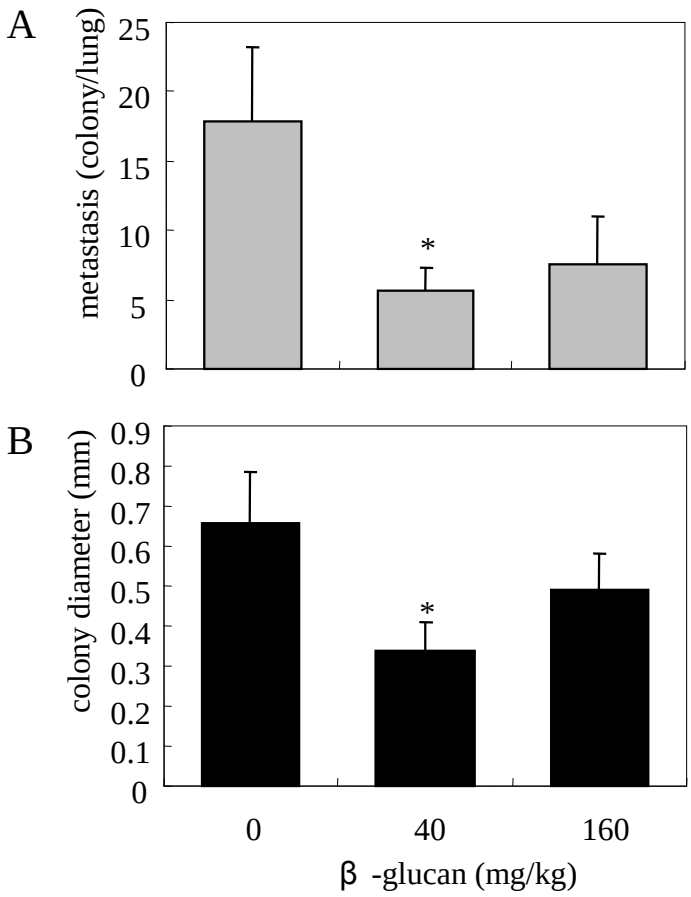

Figure 5. Effects of SBG on spontaneous metastasis. The number of pulmonary metastatic colony (A), Size of metastatic foci (B). *: $P < 0.05$ (vs control group). N = 6–7.

Effects of SBG on proliferation, migration, and capillary morphogenesis of HUVECs

To investigate possible mechanisms of SBG action against neovascularization, I examined the effect of SBG on HUVECs *in vitro*. The cells were treated with SBG and assessed for proliferation, migration, and capillary tube formation. Despite high dose application of SBG, none of the above mentioned properties of HUVECs were affected (Figure 6).

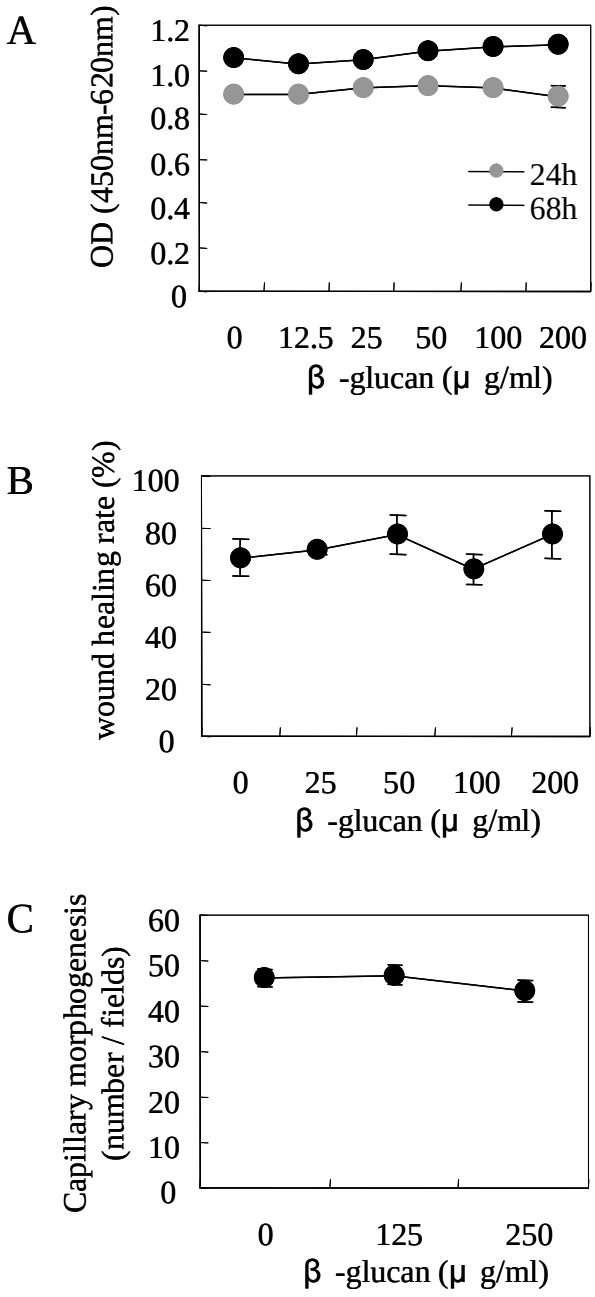

Figure 6. Effects of SBG on HUVECs. Proliferation (A), wound healing rate (B) and capillary morphogenesis (C). N = 3–8.

DISCUSSION

S. crispa is an edible and medicinal mushroom containing large amount of β-1,3-D-glucan. *S. crispa* is known to show antitumor activity in oral administration.[1)] β-1,3-D-Glucan of *S. crispa* is reported to act as an antitumor agent in tumor-bearing mice

when injected intraperitoneally and a stimulator of hematopoiesis on intraperitoneal and oral administration *in vivo*.[2,8–10] Moreover, the glucan also enhances the cytokine production in mice spleen cells and human PBMC.[5–7]

It is well accepted that the biological effects of β-D-glucan depend on its primary structures. Although β-D-glucan from *S. crispa*, SBG was structurally the same as β-D-glucan from *Schizophyllum commune*, SPG, of which major structural unit is β-(1,3)-D-glucan backbone with single β-(1,6)-D-glucosyl side branching units every three residues, strangely, SBG but not SPG induces cytokine production from bone marrow-derived dendritic cells (BMDCs).[3]

Hence, I tried to elucidate the primary structure of SBG by chemical analysis, and found β-(1,2)-D-glucosyl side branching units newly, except for β-(1,6)-D-glucosyl side branching units. The difference of the biological activity between SBG and SPG might be derived from the existence β-(1,2)-D-glucosyl side branching units.

The antitumor mechanisms of *S. crispa*, except for the immuno-modulating actions, have not been well studied. Hence, I tried to elucidate possible mechanisms of anti-angiogenic potential of SBG using two different animal models. In this study, I investigated the effect of β-D-glucan from *S. crispa* on angiogenesis, particularly by oral administration, since there are very few reports demonstrating that orally administered glucan inhibits neovascularization.

First, I demonstrated the anti-angiogenic activity of SBG in the DAS system. The suppressive effect of VEGF-induced neovascularization was further confirmed in Matrigel plug assay which enables sustained release of VEGF *in vivo*. I further analyzed the anti-metastatic effects of SBG, since angiogenesis is an essential event for tumor cells to grow *in vivo* and to spread by metastasis. In B16-BL6-bearing mice, tumor growth rate was significantly retarded by preventive administration of SBG. The number of lung metastatic colonies and growth of these metastatic foci were also significantly suppressed. In this experiment, dose-dependency of anti-metastatic effects was not observed. However, Miura T., *et al*.[27] has reported that overdose application of β-glucan (sonifilan) fails in expressing antitumor activity. Hence, appropriate dosage should be essential for long-term use of SBG.

All animal studies in my study, SBG was pre-administered before surgical treatment

of mice to estimate significant effects of SBG. I did not evaluate the effects of SBG without pre-administration. However, SBG is expected to be effective on angiogenesis and metastasis without pre-administration, because my previous study showed post-administration of powdered SC toward tumor-bearing mice was also effective to suppress tumor growth.

Several mushrooms have been reported to show anti-angiogenic and anti-metastatic effects, such as *Agaricus blazei*, *Ganoderma lucidum*, *Cordyceps militaris*, and *Coriolus vesicolor*, and their active substances have been identified as triterpenoid, ergosterol, and pyroglutamate etc.[23,25,26,28–30] Fungal protein-bound polysaccharide derived from *C. vesicolor* (PSK) was also reported to act as an anti-angiogenic or anti-metastatic agent.[31] However, PSK is mainly constituted of β-1,4-bond glucan main chain having β-1,3 and β-1,6 bond side chain binding to a protein moiety.[32] Therefore, it was considered that SBG is a novel anti-angiogenic and anti-metastatic agent being particularly effective in oral administration.

The mechanisms of anti-angiogenic actions of SBG as well as PSK remain to be clarified; the possibilities include inhibitory effects of proliferation, migration and capillary morphogenesis of vascular endothelial cells. I therefore examined the direct actions of SBG on HUVECs. However, no significant effects were shown. Since β-glucan is a kind of dietary fiber and highly resistant against digestive enzyme, metabolite of β-glucan is not likely to contribute these effects directly. This observation suggests that SBG suppresses angiogenesis of vascular endothelial cells by an indirect mode of action.

β-1,3-D-Glucan derived from SC is reported to stimulate IFN-γ and IL-12p70 production from mice spleen cells.[4,5] Several cytokines such as TNF-α, IFN-α, IFN-γ, IL-12, and interferon-inducible protein 10 (IP-10) are known anti-angiogenic factors.[33–36] In the present study, it might be possible that such anti-angiogenic proteins contribute to the action of SBG, because IFN-γ production from splenic lymphocytes of SBG-fed mice was relatively increased compared to the control group (Figure 7). When lymphocytes were stimulated by SBG, IFN-γ secretion level was highest in 40 mg/kg/day group. It was correlated with the inhibition activity of metastasis. The possibility that orally administered SBG leads to enhanced production of anti-angiogenic proteins needs further investigation.

In conclusion, my results indicate that oral administration of β-D-glucan purified

from SC, which has β-(1,6) and β-(1,2)-D-glucosyl side branching units, in mice showed anti-angiogenic and anti-metastatic effects. My observations suggest that the anti-angiogenic effect of β-glucan purified from *S. crispa* may be involved in the anti-metastatic effects as well as antitumor effects.

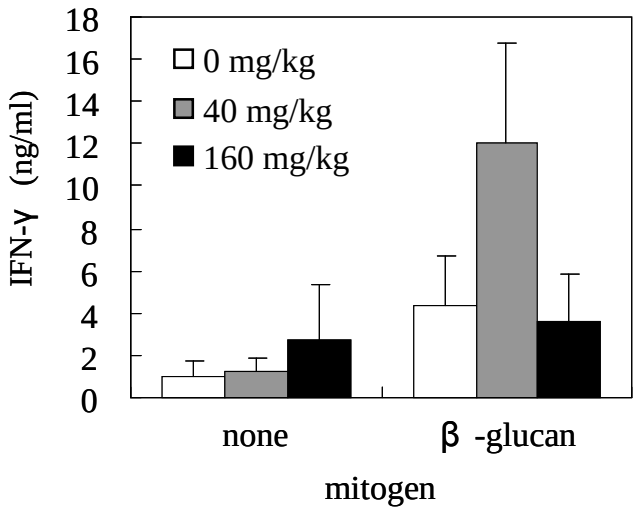

Figure 7. IFN-γ secretion from splenic lymphocytes derived from SBG-fed mice. Splenic lymphocytes were cultured with or without 100 μg/ml SBG for 72 h. N = 4.

ABSTRACT

Sparassis crispa, Hanabiratake in Japanese, is an edible mushroom with medicinal properties, that contains more than 40% β-D-glucan. It was concluded from results of the methylation study that β-D-glucan from *S. crispa* (SBG) was composed of a backbone of β-(1,3) -linked D-glucopyranosyl residues, and had β-D-glucopyranosyl groups joined through *O*-6 and *O*-2 of D-glucose of the backbone. I purified SBG and investigated its anti-angiogenic functions and anti-metastatic effects on neoplasm using different animal models. The oral administration of the purified SBG suppressed B16-F10 cell-induced angiogenesis in the dorsal air sac system using female ICR mice as well as vascular endothelial growth factor-induced neovascularization in the Matrigel plug assay using female C57BL/6J mice. Furthermore, it suppressed the growth and numbers of the metastatic tumor foci in lung, along with the primary tumor growth in the spontaneous metastatic model using female C57BL/6J mice. From these results, it is apparent that the oral administration of SBG results in suppressive effect on tumor growth and metastasis in lung through the inhibition of tumor induced-angiogenesis. These effects are not a result of direct action on the endothelial cells because cell growth, migration and capillary-like tube formation were not affected in the human umbilical vein endothelial cells by SBG application. This is the first report showing that the oral administration of SBG is capable of suppressing angiogenesis and metastasis.

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Chapter 3 Antitumor Activities of the Low Molecular Weight Fraction Derived from the Cultured Fruit Body of *Sparassis crispa* in Tumor-Bearing Mice

INTRODUCTION

Many studies about the antitumor activity of edible mushrooms have focused particularly on the effects of β-glucan. I investigated the antitumor effects of β-1,3-D-glucan (SBG) purified from *Sparassis crispa*.

However, to date, only few studies have investigated the antitumor effects of compounds other than β-glucan in edible mushrooms.[1-4] In particular, the antitumor activities of low molecular weight components from *S. crispa* remain to be clarified.

In this study, I investigated the antitumor effects of low molecular weight fraction (containing no β-glucan) isolated from hot water (H_2O) extract (FHL) of the cultured fruit body of *S. crispa*. Further, I examined the effect of oral administration of FHL on the production of cytokines by murine splenocytes, which were obtained from mice fed with or without FHL. In addition, I examined the anti-angiogenic effect of FHL by using the dorsal air sac (DAS) assay.

MATERIALS and METHODS

Materials

A regenerated cellulose membrane with a molecular weight cutoff of 6,000–8,000 (Spectra Pore; MWCO 6,000-8,000) was purchased from Funakoshi (Tokyo, Japan). Eagle's minimal essential medium (EMEM) and RPMI 1640 medium were obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and antibiotic-antimycotic solutions (100×) containing 10,000 units/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin B in phosphate-buffered saline (PBS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fetal bovine serum

(FBS) was purchased from Gibco BRL (Auckland, New Zealand). Diffusion chamber ring, MF cement, and 13-mm circular membrane filters were obtained from Millipore (Tokyo, Japan).

Cells

Sarcoma 180 cells (DS Pharma Biomedical Co., Ltd., Osaka, Japan) were cultured in EMEM supplemented with 10% (v/v) FBS and 1× antibiotic-antimycotic solution. The highly metastatic B16-F10 cells (America Type Culture Collection) were maintained in DMEM supplemented with 10% (v/v) FBS and 1× antibiotic-antimycotic solution.

Animals

Female ICR mice (4 or 5 weeks old) were obtained from Japan SLC (Shizuoka, Japan), and Clea Japan (Osaka, Japan). They were housed in a room maintained at 23°C ± 3°C with a 12-h light/dark cycle with free access to water and food (CRF-1; Oriental Yeast Co., LTD., Tokyo, Japan or Labo MR stock; Nosan Corporation, Kanagawa, Japan). The mice were treated according to the ethical guidelines prescribed by the Animal Study of Unitika Ltd.

Preparation of FHL from S. crispa

The dry powder of the fruit body of *S. crispa* (50 g) was extracted twice with 2 L of hot distilled water (100°C, 2 h) in succession. The extract was lyophilized, and the residue was dissolved in a small amount of distilled water. After exhaustive dialysis was performed using a dialysis membrane (MW cutoff, 6,000-8,000) at 4°C against distilled water, FHL was obtained by freeze-drying the outer dialysis solution.

The total amount of sugars in FHL was measured using phenol-sulfuric acid method with glucose as the reference. The levels of glucose were measured using Glucose Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The level of trehalose was determined using high-performance liquid chromatography (HPLC) at the Japan Food Research Laboratories. Protein content in FHL was measured using Bio-Rad protein assay (Bio-Rad,

California, US) with bovine serum albumin as the reference.

Molecular weight of FHL was analyzed using an HPLC system equipped with an Ultrahydrogel Column (Waters, Connecticut, US). A 0.5 M phosphate buffer (pH, 11) was used as the mobile phase at a flow rate of 0.5 mL/min, and the differential refractive index was detected.

The antitumor effect of FHL

A sarcoma 180 cell suspension was subcutaneously implanted (2×10^6 viable cells) into the right upper extremity of each mouse after 1 week of acclimatization. After 1 week, a water solution containing FHL (corresponding to 30 mg/kg body weight) was administered using a stomach tube. Oral administration was performed daily for 15 days. Tumor size was measured twice a week with a caliper, and tumor volume was calculated using the following formula: $a^2b/2$, where a is the smallest and b is the largest diameter in millimeters.

Effect of FHL on immunomodulative activity in tumor-bearing mice

The effects of oral administration of FHL on the production of cytokines were investigated using a culture system with splenocytes derived from above-mentioned tumor-bearing mice administered FHL. Briefly, oral administration of a water solution containing FHL (corresponding to 30 mg/kg body weight) was performed daily for 7 days from 14 days after inoculation. Mice were killed on day 21 to obtain splenocytes. After depletion of erythrocytes, the cells (5×10^5/mL) were cultured in 0.2-mL of RPMI 1640 medium supplemented with 10% (v/v) FBS and 1× antibiotic-antimycotic solution. Culture supernatants were collected from the wells at 24 h after seeding cells. The levels of interferon γ (IFN-γ) and interleukin 4 (IL-4) were measured using Mouse IFN-γ ELISA kit and Mouse IL-4 ELISA kit (Pierce Biotechnology, Rockford, USA), respectively.

Tumor-induced angiogenesis in the DAS assay

DAS assay was performed according to the method described by Oikawa T., et al.[5] with slight modification. Briefly, both sides of the diffusion chamber ring were covered with

membrane filters, and the resulting chambers were filled with B16-F10 cells (1.5×10^6 cells) in 150 μL of PBS. The B16-F10 cells in each chamber were implanted into the dorsal air sac of female ICR mice on day 0. The negative control group was implanted with the PBS-containing chamber. Water or FHL (30 mg/kg body weight) was orally administered once a day from 7 days before to 6 days after day 0 for a total of 14 days. On day 7 after implantation, each mouse was killed and tumor cell-induced angiogenesis was observed at the site of implantation. The number of newly formed blood vessels > 3 mm in length with a characteristic zigzag shape was counted.

Statistical analysis

All values are presented as the means ± standard deviation (SD). Statistical comparisons were analyzed using either Student's *t*-test or Dunnett's test. P values less than 5% ($p < 0.05$) and less than 10% ($p < 0.10$) were considered significant and marginally significant, respectively.

RESULTS

Preparation and characterization of FHL

The weight of FHL was 6.2 g, and its yield was 12.4% (w/w). The major sugar in FHL was trehalose. The protein content of FHL was less than 0.1% (Table 1).

Table 1. Approximate composition of low molecular weight fraction (containing no β-glucan) isolated from hot water extract (per 100 g dry sample)

Components	Amount (g)
Total sugar[1)]	22.15
Glucose[2)]	3.67
Trehalose[3)]	15.90
Total protein[4)]	0.04

[1)] Phenol-sulfuric acid method [2)] Mutarotase-GOD method

[3)]HPLC method [4)]Bradford method

Gel chromatography showed that molecular weight of FHL was less than 8,000 (data not shown). In addition, the physicochemical properties of FHL were examined using Congo Red-induced metachromasy, which is a well-known property of a high molecular weight and gel-forming β-1,3-D-glucan.[6)] FHL did not show metachromasy with Congo Red in that the absorption maximum did not shift to a shorter wavelength in alkaline conditions (data not shown).

Antitumor activity of FHL

Administration of consecutive doses of FHL tended to suppress tumor growth (Figure 1). Data of tumor volume on week 5 after inoculation showed 42.4% inhibition of tumor growth.

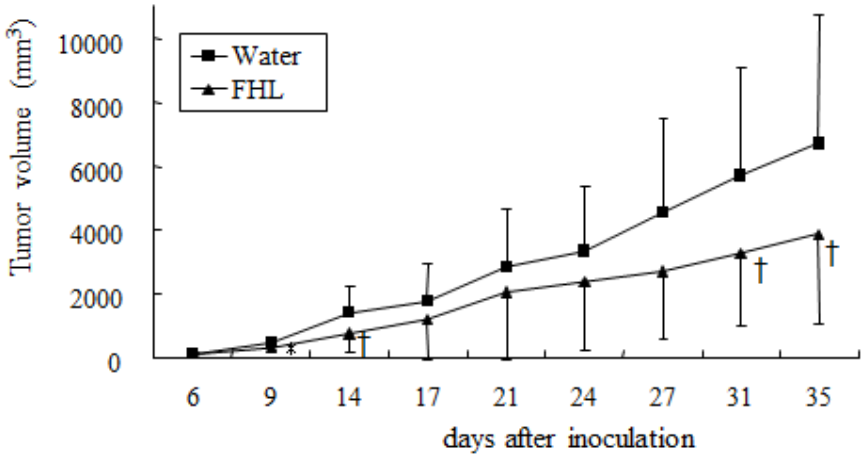

Figure 1. Changes in tumor size in mice orally administered with low molecular weight fraction (containing no β-glucan) isolated from hot water extract (FHL).
The mice were subcutaneously implanted with 2×10^6 sarcoma 180 tumor cells. An aqueous solution containing FHL (corresponding to 30 mg/kg body weight) was orally administered daily to the mice implanted with tumor cells for 15 days from 7 days after inoculation. Tumor size was measured twice a week with a caliper, and the tumor volume was calculated. Values are means ± SD of 10 mice per group for each time point. Student's *t*-test was used for

statistical analysis. *$p < 0.05$, †$p < 0.10$ compared to the control (water) group.

Effect of FHL on immunomodulative activity in tumor-bearing mice

To examine the immunomodulative activity of oral administration of FHL, splenocytes obtained from sarcoma 180-bearing mice administered with FHL were cultured *in vitro*. The IFN-γ level in the culture supernatant of splenic lymphocytes from the FHL-treated tumor-bearing mice was significantly higher than that in the control group (Figure 2A). However, the IL-4 level in the FHL-treated mice decreased (Figure 2B).

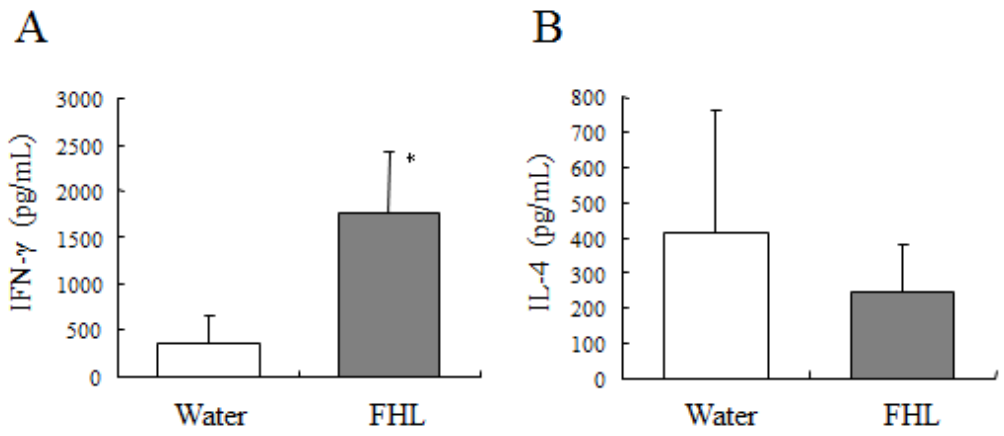

Figure 2. Effects of oral administration of low molecular weight fraction (containing no β-glucan) isolated from hot water extract (FHL) on the levels of interferon γ (IFN-γ) and interleukin 4 (IL-4) secreted by splenocytes derived from sarcoma 180-bearing mice.
IFN-γ (A) and IL-4 (B). The averages and error bars representing the SD were obtained from the data of 3 mice, and Student's *t*-test was used for statistical analysis. Control (water) group (open columns); FHL-treated group (filled columns). A significant difference of *$p < 0.05$ compared to the control group was observed.

Effects of oral administration of FHL on tumor induced angiogenesis

I examined the effects of FHL on tumor-induced neovascularization using the DAS assay. Marked induction of angiogenesis was observed after implantation of B16-F10 cells in the chamber; however, oral administration of FHL tended to inhibit the tumor-induced

angiogenesis (Figure 3).

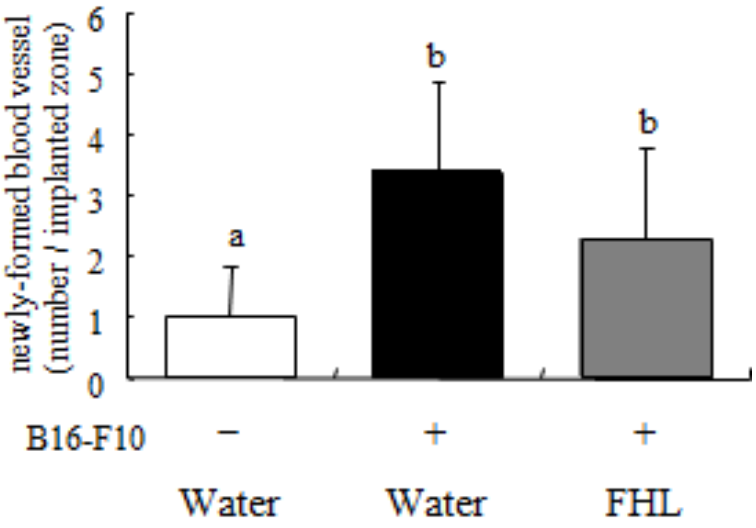

Figure 3. Effects of low molecular weight fraction (containing no β-glucan) isolated from hot water extract (FHL) on tumor-induced angiogenesis in the dorsal air sac (DAS) assay. The number of newly formed blood vessels > 3 mm length with a characteristic zigzag shape in the chamber implanted zone was indicated. The averages and error bars representing the standard deviation were obtained from the data of 7–13 mice per group, and comparisons among groups were performed using Dunnett's test. Superscript letters a and b represent significant differences in the mean values ($p < 0.05$).

DISCUSSION

Ohno et al. showed that β-1,3-D-glucan from *S. crispa* has a symmetric molecular weight distribution with an average molecular weight of *ca*. 10^6 Da.[6] Because FHL had a molecular weight cutoff of less than 8,000 and did not show metachromasy with Congo Red, it is unlikely that FHL contains β-1,3-D-glucan. Meanwhile, FHL contains a relatively large amount of trehalose. Oral administration of trehalose modified the mucosal immune responses of the small intestine in mice[7] and suppressed tumor growth in tumor-bearing mice.[8] Therefore, antitumor activities of FHL may be partly attributed to trehalose.

Generally, tumor-bearing mice tend to shift to Th2 rather than Th1 in the Th1/Th2 balance.[9] Oral administration of FHL to tumor-bearing mice suppressed tumor-growth. Furthermore, the IFN-γ (Th1-type cytokine) level in the culture supernatant of splenic lymphocytes of FHL-treated tumor-bearing mice was significantly higher than that in splenic lymphocytes of mice in a control group orally administered with water. Further, IL-4 (Th2-type cytokine) level in FHL-treated mice decreased. Thus, FHL may express antitumor activity after the induction of a Th1 response, which leads to potentiation of cytotoxic activity.

Angiogenesis plays an important role in the growth of solid tumors; thus, anti-angiogenic strategies are considered to be highly effective in cancer therapy.[10–12] Tumor-induced angiogenesis in the DAS system was weakly suppressed by administration of FHL.

These results suggest that oral administration of FHL induces antitumor activity by increasing the Th1-response, and the anti-angiogenic activity may also contribute to the antitumor activity of FHL. However, further studies are required to elucidate the active substance in FHL.

ABSTRACT

Many researches concerning antitumor activity of edible mushrooms take particular note of β-glucan. However, there are less report focusing on antitumor-components other than β-glucan. In this study, I investigated the antitumor effects of low molecular weight fraction (FHL) (containing no β-glucan) isolated from hot water extract of the cultured fruit body of *Sparassis crispa*. The oral administration of FHL to tumor-bearing mice suppressed tumor-growth. Furthermore, the interferon (IFN)-γ level in the culture supernatant of splenic lymphocytes from the FHL-fed tumor-bearing mice was significantly increased compared to control group, and the interleukin (IL)-4 level was decreased. Tumor-induced angiogenesis was suppressed by the FHL administration in the dorsal air sac (DAS) system. These results suggested that the oral administration of FHL showed antitumor activity through the enhancement of the Th1-response of tumor-bearing mice. Anti-angiogenic activity of FHL

may contribute to the antitumor activity of FHL.

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Chapter 4 Novel Phthalide Compounds from *Sparassis crispa*, Hanabiratakelide A–C, Exhibiting Anti-cancer Related Activity

INTRODUCTION

Sparassis crispa, known as Hanabiratake in Japanese, is an edible mushroom with various medicinal properties that has recently become cultivable in Japan. *S. crispa* grows primarily on the stumps of coniferous trees and is abundant in northern temperate zones throughout the world.[1)] More than 40% of *S. crispa* is composed of β-D-glucan, a polysaccharide of D-glucose comprising a β-(1,3)-D-glucan backbone with a single β-(1,6)- or β-(1,2)-D-glucosyl side branch at every third residue.[2–4)] *S. crispa* has been reported to have many biological effects such as tumor-suppressing effects,[2,4,5)] effects in improving natural killer cell activity,[5)] anti-angiogenic effects,[4)] anti-allergic effects,[6)] wound-healing effects,[7)] and effects in enhancing hematopoietic responses.[2,8)] In addition, a recent study suggested that the administration of β-D-glucan from *S. crispa* could be an effective treatment strategy for cancer patients.[9)]

In continuing search for biologically active compounds from fungi,[10)] I have initiated chemical studies of *S. crispa*. Previous studies have reported the isolation of a sesquiterpenoid[11)] 2 maleic acid derivatives,[12)] and a benzoate derivative—sparassol.[13)] In this chapter, I describe the isolation and structural elucidation of hanabiratakelides A–C (**1**–**3**), primarily by extensive NMR experiments, along with 3 known phthalides (**4**–**6**),[14,15)] 2 unsaturated fatty acids,[16,17)] and ubiquinone-9.[18)] The biological activity of the isolated compounds, especially **1**–**3**, are also indicated.

Reactive oxygen and nitrogen oxide species (RONS), such as hydroxyl radical (OH), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxynitrite ($ONOO^-$), are generated by respiring cells and act as signal transduction mediators within cells. They play important roles in numerous biological processes, such as cell differentiation, immune activation, and apoptosis. However, excess amounts of these molecules can be

harmful to cells and are associated with the pathogenesis of cancer, atherosclerosis, inflammation, and other such disorders.[19,20] Prostaglandin E2 (PGE2) is a bioactive compound synthesized in the arachidonic acid cascade. It has been reported that overproduction of PGE2 contributes to tumor progression by activating signaling pathways that stimulate cell proliferation, migration, and angiogenesis.[21]

Most RONS and PGE2 are generated by activated immune leukocytes. Therefore, suppression of over-activated leukocytes is considered beneficial for preventing oxidation- and inflammation-related diseases, including cancer.[19–22] Several phytochemicals have been shown to have anti-oxidant or anti-inflammatory effects. Some of these, such as curcumin and epigallocatechin gallate, are being investigated in clinical trials for cancer prevention.[23]

So far, no compounds in SC have been reported to have anti-oxidant or anti-inflammatory activity. In this study, I purified 3 novel phthalides and tested them for anti-oxidant, anti-inflammatory, and anti-tumor properties *in vitro*. Since compounds possessing these properties commonly also have cancer-preventive activity,[19–23] I also assessed *S. crispa* for possible anti-carcinogenic activity *in vivo* in rats.

MATERIALS and METHODS

Fungus

The mycelia of *S. crispa* were grown in a solid medium containing coniferous sawdust as a base material, and the fruit bodies were harvested in Unitika Ltd., Aichi, Japan, in 2007.

Extraction and isolation

The dry powder of the fruit body of *S. crispa* (1.0 kg) was exhaustively extracted with MeOH at room temperature for 3 months. The MeOH extract residue was partitioned between EtOAc and H_2O. The EtOAc-soluble portion (41.6 g) was subjected to silica gel column chromatography with hexane-AcOEt-MeOH (9:1:0 → 0:9:1) to provide fractions 1–9. Fraction 1 (13.7 g) was subjected to preparative TLC [hexane-AcOEt (7.5:2.5)] to provide

ubiquinone-9 (102.8 mg).[18] Fraction 6 (1.37 g) and fraction 7 (1.47 g) were purified by preparative HPLC (15–30% MeOH) to provide hanabiratakelide C (**3**, 94.1 mg); compound **4** (9.5 mg)[14] was isolated from fraction 6, and compound **5** (6.0 mg)[15] from fraction 7. Fraction 8 (1.32 g) was purified by preparative HPLC (70–100% MeOH) to yield filoboletic acid (61.1 mg)[16] and 8,9-dihydroxy-10*E*,12*Z*-octadecadienoic acid (42.0 mg).[17] The residue of fraction 8 was further purified by preparative HPLC (30–50 % MeOH) to provide hanabiratakelides A (**1**, 19.0 mg) and B (**2**, 47.3 mg) and compound **6** (11.5 mg).[15]

Hanabiratakelide A (1): A colorless amorphous solid, with mp 236–238°C. FT-IR (dry film) cm^{-1}: 3165 (OH), 1716 (C=O), 1622, 1455 (aromatic). UV λ_{max} (MeOH) nm (log ε): 260 (3.78), 309 (3.66). ^{1}H-NMR (600 MHz, C_5D_5N) δ: 3.78 (3H, s, OMe), 5.40 (2H, br s, H_2-3), 6.85 (1H, br s, H-7). ^{13}C-NMR (150 MHz, C_5D_5N) δ: 56.1 (q, OMe), 67.3 (t, C-3), 101.5 (d, C-7), 104.7 (s, C-7a), 134.5 (s, C-3a), 136.7 (s, C-4), 153.4 (s, C-6), 154.6 (s, C-5), 169.3 (s, C-1). High-resolution electron ion mass spectrum (HR-CI-MS) *m/z*: 197.0431 (calculated for $C_9H_9O_5$: 197.0422).

Hanabiratakelide B (2): A colorless amorphous solid, with mp 202–204°C. FT-IR (dry film) cm^{-1}: 3235 (OH), 1708 (C=O), 1603, 1473 (aromatic). UV λ_{max} (MeOH) nm (log ε): 235 (3.41), 264.5 (3.41), 300 (3.13). ^{1}H-NMR (600 MHz, C_5D_5N) δ: 4.17 (3H, s, OMe), 5.14 (2H, br s, H_2-3), 6.98 (1H, br s, H-4). ^{13}C-NMR (150 MHz, C_5D_5N) δ: 62.0 (q, OMe), 69.0 (t, C-3), 104.8 (d, C-4), C-109.1 (s, C-7a), 140.0 (s, C-5), 140.8 (s, C-3a), 147.0 (s, C-6), 155.9 (s, C-7), 169.8 (s, C-1). HR-CI-MS *m/z*: 197.0411 (calculated for $C_9H_9O_5$: 197.0422).

Hanabiratakelide C (3): An amorphous solid, with mp 122–124°C. FT-IR (dry film) cm^{-1}: 3350 (OH), 1729 (C=O), 1624, 1518 (aromatic). UV λ_{max} (MeOH) nm (log ε): 275 (4.19), 302 (3.83). ^{1}H-NMR (600 MHz, C_5D_5N) δ: 4.11 (3H, s, OMe), 5.41 (2H, s, H_2-3). ^{13}C-NMR (150 MHz, C_5D_5N) δ: 62.2 (q, OMe), 67.7 (t, C-3), 108.0 (s, C-7a), 126.1 (s, C-3a), 137.6 (s, C-4), 140.5 (s, C-6), 140.9 (s, C-5), 143.3 (s, C-7), 170.2 (s, C-1). HR-CI-MS *m/z*: 213.0411 (calculated for $C_9H_9O_6$: 213.0422).

General experimental procedures

Melting points were determined with a Yanagimoto micro melting point apparatus.

IR spectra were measured on a Jasco FT/IR-5300 instrument, and UV spectra were recorded using a Shimadzu UV-6000 spectrophotometer. NMR spectra were recorded on a Varian Unity 600 spectrometer. The chemical shifts are given in δ (ppm) in C_5D_5N solution, using tetramethylsilane as an internal standard. NMR experiments included correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple-bond correlation (HMBC), and rotating-frame Overhauser enhancement spectroscopy (ROESY). Coupling constants (*J* values) are given in hertz (Hz). HR-CI-MS were measured on a JEOL JMS-700 MS station.

Materials

Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium were obtained from Wako (Tokyo, Japan). Antibiotic-antimycotic solution (100×) containing 10,000 units/ml penicillin, 10 mg/ml streptomycin and 25 μg/ml amphotericin B in phosphate-buffered saline (PBS) was purchased from Wako (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Gibco BRL (Auckland, New Zealand). Lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, MO, USA).

Cells

The human colon cancer Caco-2 cells, kindly provided by Dr. Shimizu from the University of Tokyo, were maintained in DMEM supplemented with 10% (v/v) FBS and 1× Antibiotic-Antimycotic solution. The murine colon cancer colon-26 cells and mouse macrophage-like RAW264 cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer of Tohoku University, and the RIKEN BioResource Center, respectively. These cell lines were maintained in RPMI-1640 supplemented with 10% (v/v) FBS and 1× Antibiotic-Antimycotic solution.

Superoxide dismutase (SOD)-like activity

SOD-like activity was determined according to the method of Ukeda[24] using an SOD Assay Kit-WST (Dojindo Laboratory, Kumamoto). A test sample was dissolved in DMSO to a

final DMSO concentration of 0.8% (v/v). The results are expressed as an IC_{50}, which is the drug concentration required to inhibit enzyme activity to 50% of that of an untreated control.

PGE2 and NO-induction in RAW264 cells

Analyses of the anti-inflammatory potentials of the 3 compounds were according to the methods of Han *et al.* and Lee *et al.*[16,17] Briefly, RAW264 cells were cultured in a 96-multiwell plate at 5×10^5 cells/ml in the presence of LPS (0.1 μg/ml) and the purified compounds. Culture supernatants were collected from the wells at 24 h after seeding cells. PGE2 and NO were measured by ELISA (R&D Systems, Minneapolis, MN, U.S.A.) and the Griess reagent system (Promega, Madison, WI, U.S.A.), respectively.

Colon cancer cell growth assay in vitro

Colon cancer cell lines were seeded into a 96-multiwell culture plate at 3×10^4 cells/ml, and the isolated compounds were added. Cell proliferation was determined using a cell counting kit (CCK-8, Dojindo Laboratory) at 72 h post-seeding.

Animals

Female F344/N rats (5 weeks old) were obtained from Japan SLC (Shizuoka, Japan). They were housed in a room maintained at 24 ± 1°C with a 12 h light-dark cycle. Powdered *S. crispa* was mixed with AIN-76 diet (Japan Clea, Osaka) at 0.3, 1.0, and 3.0% (w/w) of the total diet and fed to rats. Control rats were fed AIN-76 diet only. The animals were treated according to the ethical guidelines prescribed by the Animal Study committee of Unitika Ltd.

Induction of aberrant crypt foci (ACF) by azoxymethane (AOM)[18]

AOM solution (15 mg/kg) was subcutaneously injected at 1 and 2 weeks after beginning the feeding with the experimental or control diet. Three weeks after the second AOM injection, each rat was sacrificed and its colon was excised. Excised colons were cut like a sheet, washed with PBS, and fixed with 20% formaldehyde solution for more than 72 h. The numbers of ACF and total aberrant crypts (ACs) per colon were counted using a

stereomicroscope after staining with 0.2% methylene blue.

Statistical analysis

All results are given as means ± SD. Comparisons between means were analyzed by Dunnett's test. Differences were considered significant at $P < 0.05$.

RESULTS

Chemical characterizations of isolated compounds

The chemical structures of the isolated compounds (**1**–**6**) are shown in Figure 1. Hanabiratakelide A (**1**) was obtained as a colorless amorphous solid and gave an $[M + H]^+$ peak at *m/z* 197.0431 on its HR-CI-MS. This corresponded to the molecular formula $C_9H_8O_5$, which required 6 unsaturation equivalents. The IR spectrum of **1** contained absorption bands at 3165 (OH), 1716 (C=O), and at 1622 and 1455 (aromatic) cm^{-1}. Also, the presence of an aromatic ring was supported by UV data (λ_{max} 260, 3.09 nm). In the ^{13}C-NMR spectrum of **1**, 9 resonances attributable to 1 methoxy (δ56.1), 1 oxymethylene (δ67.3), 6 olefinic (δ 101.5 (d), 104.7 (s), 134.5 (s), 136.7 (s), 153.4 (s), 154.6 (s)] and 1 carbonyl carbon (δ 169.3) were evident. The ^{1}H-NMR spectrum of **1** clearly indicated signals corresponding to methoxyl at δ 3.78 (3H, s), oxymethylene at δ 5.40 (2H, br s), and an aromatic proton at δ 6.85 (1H, br s). As the data could account for 6 of the hydrogen atoms within **1,** it was evident that the remaining 2 must be present as parts of OH groups, a deduction that was also supported by the IR spectrum. Further, the remaining 2 unsaturation equivalents of **1** could be satisfied by constructing 2 rings.

The gross structure of **1** was determined by analyzing 2D NMR data, including COSY, HMQC, HMBC, and ROESY. The COSY spectrum revealed a homoallylic coupling between H_2-3 (δ 5.40) and H-7 (δ 6.85). The HMBC spectrum of **1** contained cross peaks from H_2-3 to C-1 (δ 169.3), C-3a (δ 134.5), C-4 (δ 136.7), and C-7a (δ 104.7); from H-7 (δ 6.85) to C-1, C-3a, C-5 δ 154.6), and C-6 (δ 153.4); and from OMe (δ 3.78) to C-6. Furthermore, the nuclear Overhauser effect (NOE) was observed between H-7 and the methoxy signal at C-6.

These findings enabled me to construct the structure of **1** as a phthalide compound with hydroxyl groups at C-4 and C-5, and a methoxy group at C-6 as its substituents. Thus, the structure of hanabiratakelide A was established as **1**.

Hanabiratakelide B (**2**) was obtained as a colorless amorphous solid and showed an $[M + H]^+$ peak at *m/z* 197.0411 in its HR-CI-MS, indicative of a molecular formula $C_9H_8O_5$ and requiring 6 unsaturation equivalents. The IR spectrum of **2** exhibited absorption bands due to hydroxyl, carbonyl, and aromatic functions. NMR data of **2** showed the presence of the same structure fragments as those of **1**, such as 1 benzylic methylene, 1 penta-substituted benzene ring, 1 carbonyl and 1 downfield shifted methoxy group (δ_H 4.17, δ_C 62.0), suggesting that it was sandwiched between the oxygen groups. The upfield shift of H_2-3 (δ 5.14) in **2** by comparison with that of **1** (δ 5.40) suggested that C-4 was not substituted with hydroxyl or methoxy groups. The COSY spectrum revealed the connectivity of an allyl coupling between H_2-3 (δ 5.14, br s) and aromatic proton H-4 (δ 6.98, br s). HMBC showed long-range correlations from H_2-3 to C-1 (δ 169.8), C-3a, C-4 (δ 104.8), and C-7a (δ 109.1); from H-4 (δ 6.98) to C-5 (δ 140.0), C-6 (δ 147.0), and C-7a; and from OMe (δ 4.17) to C-6. These data aided with deriving the structure of **2** as a phthalide compound with hydroxyl groups at C-5 and C-7 and a methoxy group at C-6 as its substituents, which was further confirmed by the observation of NOE between H_2-3 and H-4. Thus, the structure of hanabiratakelide B was established as **2**.

Hanabiratakelide C (**3**) had the molecular formula $C_9H_8O_6$ in its HR-CI-MS, requiring 6 unsaturation equivalents like those of **1** and **2**. ^{1}H-NMR data of **3** were indicative of the presence of a benzylic methylene at δ 5.41 (2H, s), and a downfield shifted methoxy group at δ 4.11 (3H, s). However, the characteristic aromatic proton in the downfield was absent. ^{13}C-NMR data of **3** contained structure fragments including 1 methoxy, 1 benzylic methylene, 1 carbonyl carbon and a fully substituted benzene ring. The HMBC spectrum of **3** contained cross peaks from H_2-3 (δ 5.41) to C-1 (δ 170.2), C-3a (δ 126.1), C-4 (δ 137.6), C-7 (δ 143.3), and C-7a (δ 108.0); and from OMe (δ 4.11) to C-7 (δ 143.3). However, in the ROESY spectrum, NOE was not observed. On the basis of these findings and the reported NMR data for analogous compounds,[14,15] the structure of hanabiratakelide C was formulated

as **3**.

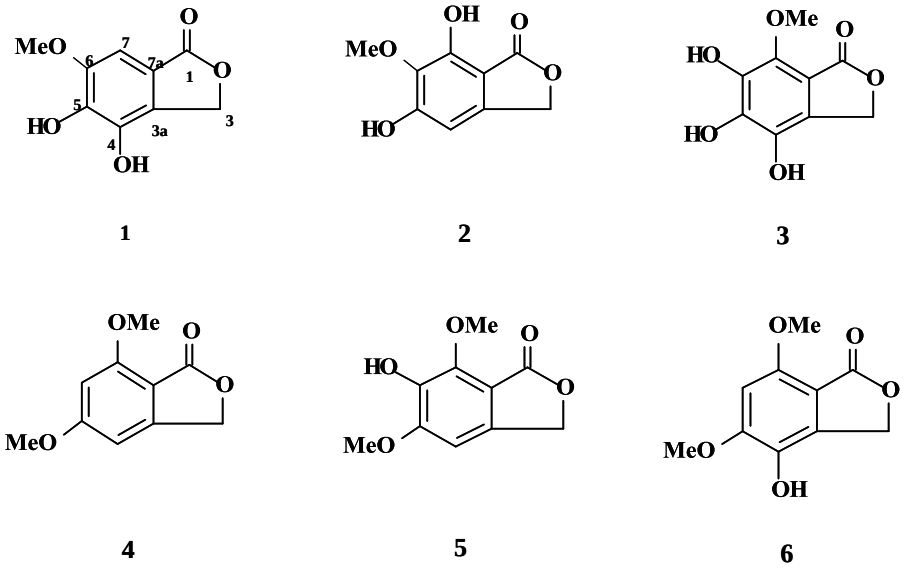

Figure 1. Chemical structures of compounds (**1**–**6**) isolated from SC.

SOD-like activity

Six phthalide compounds, **1–6,** isolated from *S. crispa* were evaluated for their SOD-like activity by using SOD water soluble tetrazolium (WST)-1 methods[24] and vitamin C as a positive control. Among these, compounds **1** and **3** showed much stronger activity than vitamin C. The IC_{50} values are shown in Table 1.

Table 1. SOD-Like Activity of Compounds 1–6

Compound	**1**	**2**	**3**	**4**	**5**	**6**	vitamin C
IC_{50} (μM)	15.7	49.0	3.2	324.9	123.0	66.8	71.0

Suppression of LPS-induced PGE2 and NO production by RAW264 cells

I also assessed isolated compounds **1–3** for other anti-inflammatory properties. These compounds significantly suppressed LPS-induced PGE2 production by RAW264 cells in a dose-dependent manner. NO production by these cells was also suppressed by treatments with compounds **1** and **3** (Figure 2).

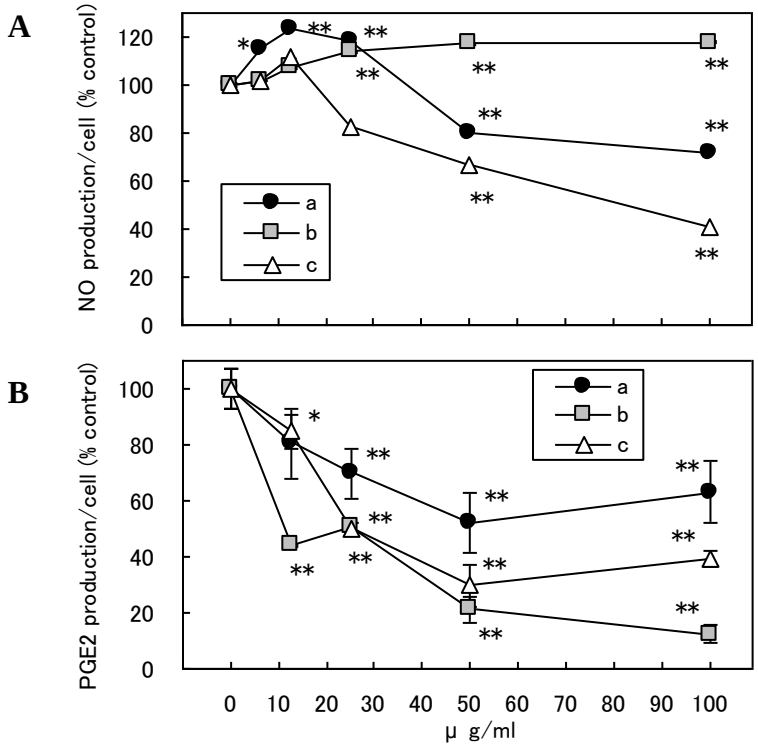

Figure 2. Anti-inflammatory activity of hanabiratakelides. LPS-stimulated NO production (A) and PGE2 release (B) are shown for RAW264 cells treated with hanabiratakelides as normalized to a control (untreated) group; values per cell were obtained by dividing by the cell number (which was determined using a cell counting kit). (a) Hanabiratakelide A, (b) Hanabiratakelide B, (c) Hanabiratakelide C, mean ± SD, N = 3, *$p < 0.05$, **$p < 0.01$.

Inhibition of colon cancer cell growth

To evaluate the anti-tumor activity of compounds **1–3**, I tested their growth inhibitory activity on colon cancer cells. Hanabiratakelide A-C significantly inhibited the growth of Caco-2 and colon-26 cells. Each compound showed dose-dependent activity, except for compound **2** in Caco-2 cells (Figure 3). The IC_{50} values of hanabiratakelide A and hanabiratakelide C in Caco-2 cells were 342 and 535 μM, respectively. Also, the IC_{50} values of hanabiratakelides A, B, and C in colon-26 cells were 96, 18, and 49 μM, respectively.

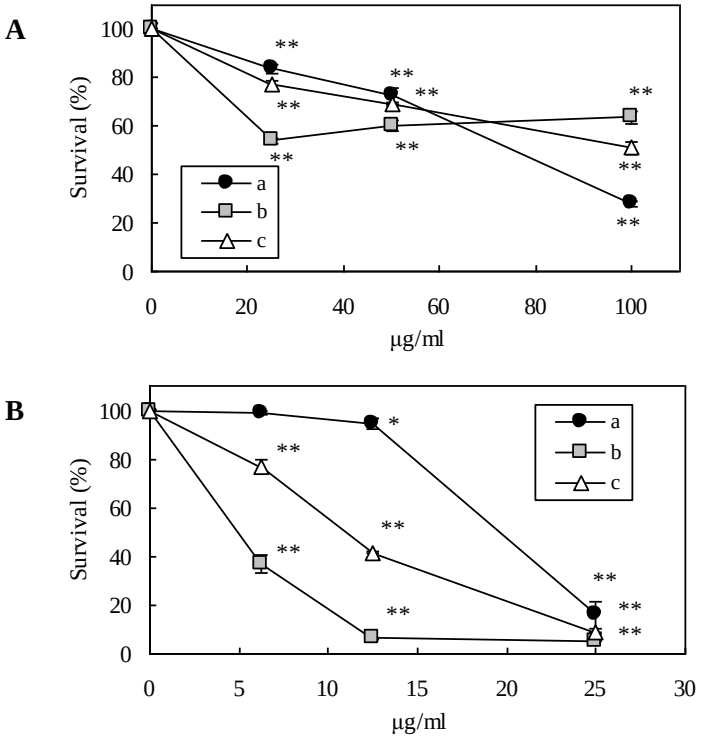

Figure 3. Cytotoxic effects of hanabiratakelides on colon cancer cell lines. Survival rates of the human colon cancer cell Caco-2 (A) and the murine colon cancer cell colon-26 (B), following treatment with the hanabiratakelides, as normalized to a control (untreated) group. (a): Hanabiratakelide A, (b): Hanabiratakelide B, (c): Hanabiratakelide C, mean ± SD, N = 4, *$p < 0.05$, **$p < 0.01$.

Preventive effects of S. crispa on AOM-induced ACF

I investigated the possible preventive effects of *S. crispa* on AOM-induced colon ACF in rats. SC feeding suppressed the malignant changes of ACF dose-dependently by 54% (0.3% group), 64% (1.0% group), and 75% (3.0% group) (Table 2).

Table 2. Suppression of AOM-induced ACF formation in F344/N rats

Experimental diet	Number of ACF/colon	Number of ACs/colon	Number of ACs/focus	Number of ACF >3ACs/colon
AIN-76	29.8 ± 11.4	51.4 ± 21.0	1.7 ± 0.1	5.0 ± 2.5
0.3% *S. crispa*†	13.7 ± 3.5*	22.3 ± 5.7**	1.6 ± 0.1	1.7 ± 0.6*
1.0% *S. crispa*†	10.8 ± 6.2**	17.5 ± 8.7**	1.7 ± 0.3	1.2 ± 1.0**
3.0% *S. crispa*†	7.4 ± 0.6**	8.7 ± 0.6**	1.2 ± 0.1*	0.3 ± 0.6**

†Powder of *S. crispa* was mixed with AIN-76 diet to give the concentrations indicated before feeding to AOM-treated rats. Mean ± SD, N = 3–6, *$p < 0.05$, **$p < 0.01$.

DISCUSSION

S. crispa is an edible mushroom with various medicinal properties and contains large amounts of β-1,3-D-glucan. Previous research on *S. crispa* has focused on its β-glucan-dependent properties.[2,4–7] Only a few studies have investigated the low-molecular weight bioactive compounds of *S. crispa*.[28] In this study, I isolated and identified 3 novel phthalides, 3 known phthalides, ubiquinone-9, and 2 unsaturated fatty-acids from the *S. crispa* fruit body. I named the 3 novel phthalides hanabiratakelide A–C and investigated their bioactive effects.

First, I found SOD-like activity in the 3 novel and 3 known phthalide compounds *in vitro*. Second, I showed inhibitory effects of the 3 hanabiratakelides on the production of NO and PGE2 by LPS-stimulated RAW264 cells. These results suggest that the 3 novel phthalides act as inhibitors of oxidative and inflammatory stress. Furthermore, cytotoxic effects of the hanabiratakelides against 2 colon cancer cell lines were demonstrated.

In general, the potential for anti-oxidant activity is often characterized by the number of phenolic hydroxyl groups in each substance. Hanabiratakelide C, having 3 hydroxyl groups,

exhibited the most significant SOD-like activity, while the activity of the other hanabiratakelides with 2 hydroxyl groups were not as strong as hanabiratakelide C. Hanabiratakelide A showed stronger activity than hanabiratakelide B. This may indicate that a phenolic hydroxyl group at C-4 enhances anti-oxidant activity. In the cell-based assay, NO appeared to be scavenged by hanabiratakelides since the patterns of inhibition of NO production were similar to the anti-oxidant effects. On the other hand, patterns of PGE2 inhibition were different from the former assays. Two other phthalides, senkyunolide A and z-ligustilide isolated from *Ligusticum chuanxiong* or *Angelica sinensis*, are also reported to have suppressive effects on LPS-activated immune cells.[29] These compounds also caused inhibition of TNF-α secretion. NO and PGE2 levels were not examined in that study. However, TNF-α secretion was inhibited at a dose in the order of μM. While further investigation is required to clarify the mode of action of hanabiratakelides, one possibility is that the hanabiratakelides act by inhibiting the activity of cyclooxygenase-2, a rate-limiting enzyme in the arachidonic acid cascade.[30]

Excess amounts of RONS and PGE2 *in vivo* are thought to be one of the risk factors for the initiation and progression of cancer. Several studies have indicated that factors suppressing the generation of RONS and PGE2 could be effective for cancer prevention.[19–23, 31, 32] The 3 novel hanabiratakelides inhibited the growth of 2 colon cancer cell lines, exhibiting IC_{50} values of 342 μM (hanabiratakelide A) and 535 μM (hanabiratakelide C) in Caco-2 cells and 96 μM (hanabiratakelide A), 18 μM (hanabiratakelide B), and 49 μM (hanabiratakelide C) in colon-26. *N*-Butylidenephthalide, senkyunolide A, and z-ligustilide are also reported to inhibit growth of the human colon cancer cell line, HT-29. The inhibitory activity (IC_{50}) of those compounds was 236.9, 54.2, and 60.6 μM, respectively, in the same order of magnitude as those observed for hanabiratakelides.[33]

ACF are early morphological changes or hyperproliferative lesions found in the colon of humans and carcinogen-treated rodents. They are also considered to be putative pre-neoplastic lesions for colon cancer.[30] Epidemiological study indicated that the frequency of colon cancer is related to dietary consumption of vegetables and fruits.[34] As described above, plant or food constituents possessing anti-oxidative or anti-inflammatory compounds

are involved in chemoprevention of cancer. In this study, feeding of *S. crispa* suppressed malignant changes of ACF in AOM-treated rats. It is also likely that hanabiratakelides in *S. crispa* might be involved in the preventive effects on carcinogenesis either directly or indirectly.

In conclusion, my results indicate that the 3 novel phthalides (hanabiratakelides) I characterized for the first time showed anti-oxidant and anti-inflammatory activity along with an inhibitory effect on tumor cell growth. Therefore, these hanabiratakelides may contribute to the cancer-preventive activity of *S. crispa*.

ABSTRACT

Sparassis crispa, known as Hanabiratake in Japanese, is an edible mushroom with various medicinal properties. Three novel phthalides, designated hanabiratakelide A (**1**), B (**2**), and C (**3**), were isolated from the *S. crispa* fruit body. In this investigation, 3 known phthalides (**4**–**6**), ubiquinone-9, and 2 known unsaturated fatty acids were also isolated. Their structures were elucidated primarily through extensive NMR experiments. The isolated compounds **1**–**6** were tested for their anti-oxidant activity. The *in vitro* superoxide dismutase-like activity of the 3 hanabiratakelides was stronger than that of vitamin C. The compounds also exerted inhibitory effects on lipopolysaccharide-stimulated nitric oxide and prostaglandin E2 production by a murine macrophage cell line, RAW264. In addition, the growth of the colon cancer cell lines Caco-2 and colon-26 was significantly inhibited by treatment with the 3 hanabiratakelides. *In vivo*, the frequency of azoxymethane-induced aberrant crypt foci was reduced in *S. crispa*-fed F344/N rats compared to rats fed a standard diet. In conclusion, 3 novel phthalides, hanabiratakelides, derived from *S. crispa* were shown to possess anti-oxidant, anti-inflammatory, and anti-tumor activity.

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Part II

Other Pharmacological Aspects of *Sparassis crispa*

Chapter 5 Effects of *Sparassis crispa* on Allergic Rhinitis in OVA-Sensitized Mice

INTRODUCTION

Type I allergies, including pollinosis, allergic rhinitis, atopic dermatitis and asthma, are characterized by an elevated production of IgE and mast cell degranulation that result in release of histamine as well as other chemical mediators of allergy.[1)] The production of IgE from B cells is regulated by T-helper (Th) cells, which have been classified into Th1 and Th2 subtype.[2)] Th2 cells synthesize IL-4, which enhances the IgE production by B cells through inducing IgE isotype class switching [3)] and the proliferation of Th2 cells.[4,5)] Th2 cells also synthesize IL-5 which enhances the IL-4-dependent IgE production.[6)] Therefore, Th2 cells and IL-4 are considered to be critical for IgE production. Conversely, IFN-γ produced by Th1 cells suppresses IgE production both by interfering with the IL-4-derived isotype class switching [7)] and by inhibiting the proliferation of Th2 cells.[8)] IL-12 produced by antigen-presenting cells, such as dendritic cells and macrophages, is known to stimulate both NK and Th1 cells to make them produce IFN-γ. These two Th1-type cytokines, IL-12 and IFN-γ, enhance the proliferation of Th1 cells.[9,10)]. It is generally accepted that enhancement of Th2-mediated immunity causes IgE-dependent allergic diseases. Therefore, properly regulating the balance between Th1- and Th2-type immune responses to heterogeneous antigens is considered to be an important mechanism in the prevention and therapy of the diseases mentioned earlier. In fact, it has been indicated that oral administration of some *Lactobacillus* can modulate the host Th1/Th2 balance and decrease serum IgE levels.[11,12)] Furthermore, oral administration of the extract from Hatakeshimeji (*Lyophyllum decastes*) mushroom can modulate the cytokine production from splenocytes and decrease serum IgE levels in NC/Nga mice.[13)]

Sparassis crispa, known as Hanabiratake in Japanese, is a tasty edible, medicinal fungus. *S. crispa* has recently become cultivable in Japan. Previously, it was reported that both serum IgE levels and the scratching amount of NC/Nga mice that were induced dermatitis by a continuous application of hapten were reduced by oral administration of *S. crispa*.[14)]

Furthermore, a branched ß-glucan from *S. crispa* can induce IFN-γ in DBA/2 mice.[15] However, the effects of oral administration of *S. crispa* on Th1/Th2 balance and allergic rhinitis have not yet been clarified.

In the present study, I examined the effects of oral administration of *S. crispa* on allergen-induced IgE and cytokines production by murine splenocytes, which were obtained from ovalbumin (OVA)-sensitized BALB/c mice fed either with or without *S. crispa*. In addition, I examined the effects of *S. crispa* on allergen-specific serum IgE levels and symptoms by the murine allergic rhinitis model.

MATERIALS and METHODS

S. crispa sample preparation

Fruiting bodies of *S. crispa* were cultivated in the Central Research Laboratories, Unitika (Kyoto, Japan). The *S. crispa* was freeze-dried and ground into ultrafine powder by a mill. The average diameter of the powder was 8 μm. The endotoxin content of the powder was below the detection limit of the Endospecy ES-50M kit (Seikagaku, Tokyo, Japan).

Animals

Female BALB/c mice (6 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). The animals were housed in an air-conditioned room maintained at 23 ± 2 °C and a relative humidity of 55 ± 15% with a 12 h light/dark cycle (8:00 - 20:00). They were given a CRF-1 diet (Oriental Yeast, Tokyo, Japan) and water *ad libitum* for at least 1 week before the experiments. Experiments were performed according to the Guidelines for the Care and Use of Experimental Animals of the Japanese Association for Laboratory Animals.

Reagents

The reagents used in the experiments include OVA (Sigma, St. Louis, MO, USA), alum (LSL Co., Tokyo, Japan) and *Bordetella pertussis* (Sigma).

Measurement of OVA-induced cytokines and OVA-specific IgE

The effects of oral administration of *S. crispa* on the antigen-induced production of cytokines and the antigen-specific IgE were investigated using a culture system with OVA-stimulated splenocytes derived from mice fed *S. crispa* as described by Fujiwara *et al* [11] with some modifications. BALB/c mice (n = 8 per group) were intraperitoneally sensitized with 20 μg of OVA absorbed onto 2 mg of alum in 0.1 ml sterile saline. Intraperitoneal injections were given twice on day 0 and day 14. They were fed a diet containing 0.25% *S. crispa* for 21 days after the first sensitization. The mean food intake was about 3 g/day per mouse, indicating that each mouse was fed approximately 7.2 mg/day of *S. crispa*. The control group was fed a normal diet without *S. crispa*. Mice were sacrificed on day 21 to obtain splenocytes. After depletion of erythrocytes, the cells (2.5×10^6/ml) were cultured with OVA (100 μg/ml) in 0.2 ml of RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with heat-inactivated fetal bovine serum (100 ml/l, Invitrogen, CA, USA), penicillin (50 U/ml), and streptomycin (50 μg/ml) in a 96-well culture plate. Supernatants were collected on day 28 for the cytokine assay and stored at –80 °C for further analysis. To monitor OVA-specific IgE production, I slightly modified the culture system of Shida *et al*.[16] Splenocytes were cultured in 1 ml culture medium with OVA for 3 days in a 48-well plate. The cultured cells were harvested, washed to remove OVA, and cultured without OVA for a further 7 days. Supernatants were collected for the OVA-specific IgE assay and stored at –80 °C for further analysis.

ELISA for OVA-specific IgE and cytokines

Measurement of OVA-specific IgE was performed by sandwich ELISA as described by Ishida *et al* [17] with some modifications. For measurement of OVA-specific IgE in mice sera and in culture supernatants, a purified goat anti-mouse IgE antibody affinity (Bethy Laboratories, Inc., Montgomery, TX, USA) was diluted with coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) to 10 μg/ml. Then, 0.1 ml of diluted antibody was soaked into each well of a 96-well Maxisorp immunoplate (Nunc, Roskilde, Denmark), and was incubated for 60 min. The microplate was washed 3 times with the washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) and blocked with blocking solution (50 mM Tris, 0.14 M

NaCl, 1% BSA, pH 8.0) for 30 min. After the microplate was washed 3 times with the washing solution, 0.1 ml of diluted samples and standard (mouse anti-OVA-specific IgE, MCA2259, Serotec, Oxford, UK) were added to each well, and incubated for 120 min. Sera samples were diluted with sample diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05 % Tween 20, pH 8.0) and supernatants of the culture were diluted with complete RPMI 1640 medium without OVA. After 3 washes with the washing solution, biotinylated OVA was diluted with sample diluent (20 μg/ml) and 0.1 ml was added to each well. Biotinylated OVA was prepared by ECL protein biotinylation module (Amersham, Buckinghamshire, UK) and the plate was incubated for 60 min. After 3 washes with the washing solution, 0.1 ml of streptavidin-peroxidase adjusted to 0.5 μg/ml with sample diluent was added to each well and incubated for 60 min. After 5 washes with the washing solution, 0.1 ml of TMB reagent (R&D systems, MN, USA) was added to the each well, followed by incubation for 30 min in the dark. Then, 0.1 ml of 2 M H_2SO_4 was added to each well and absorbance at 450 and 550 nm was measured using a microplate reader. The value of (OD_{450} - OD_{550}) was used for the specific absorbance from TMB. IFN-γ, IL-4 and IL-5 were measured using the IFN-γ ELISA kit, IL-4 ELISA kit, and IL-5 ELISA kit (Pierce Biotechnology, Rockford, USA), respectively.

Sneezing and nasal rubbing behavior induced by antigen in sensitized mice

The effects of oral administration of *S. crispa* were investigated using the allergic rhinitis model in mice as described by Kayasuga *et al* [18] with some modifications. Mice were given an intraperitoneal injection consisting of OVA (100 μg), alum (1 mg) and pertussis toxin (300 ng) in 0.1 ml sterile saline on day 0. On day 5, they were boosted by a subcutaneous injection of 1 ml of saline containing OVA (50 μg) in the back. After general sensitization, local sensitization was performed once a day from day 18 to day 53 by the instillation of OVA in saline (5 mg/ml, 10 μl/nostril) into the bilateral nasal cavities using a micropipette. They were fed either with or without *S. crispa* daily from day 25 to day 53. The number of sneezing and nasal rubbing after nasal instillation of OVA solution into the bilateral nasal cavities were counted for 30 min every week from day 25. Sera were collected on day 46 and day 53 for OVA-specific IgE quantification and stored at –80 °C for further

analysis.

Statistical analysis

Data were summarized with descriptive statistics, such as the mean and standard error of the mean. Statistical comparisons were analyzed by using either a Student's *t*-test or a Dunnett's test. A *p* value of less than 0.05 was considered significantly different.

RESULTS

Effect of S. crispa on immunomodulative activity in sensitized mice

To examine the immunomodulative activity of oral administration of *S. crispa*, splenocytes obtained from ovalbumin-sensitized BALB/c mice fed *S. crispa* were restimulated *in vitro* with the same antigen. Cytokines and OVA-IgE levels in the splenocyte culture supernatants were measured by using ELISA. Splenocytes from the *S. crispa* group showed significantly higher IFN-γ production ($p<0.01$; Figure 1A) and significantly lower IL-4 production ($p<0.01$; Figure 1B) compared to the control group. Splenocytes from the *S. crispa* group produced less IL-5 and OVA-specific IgE than the control group (Figure 1C and D), but showed no significant difference. The inhibition of Th2-type cytokine and OVA-specific IgE production seemed to be associated with the oral administration of the *S. crispa* that induced IFN-γ.

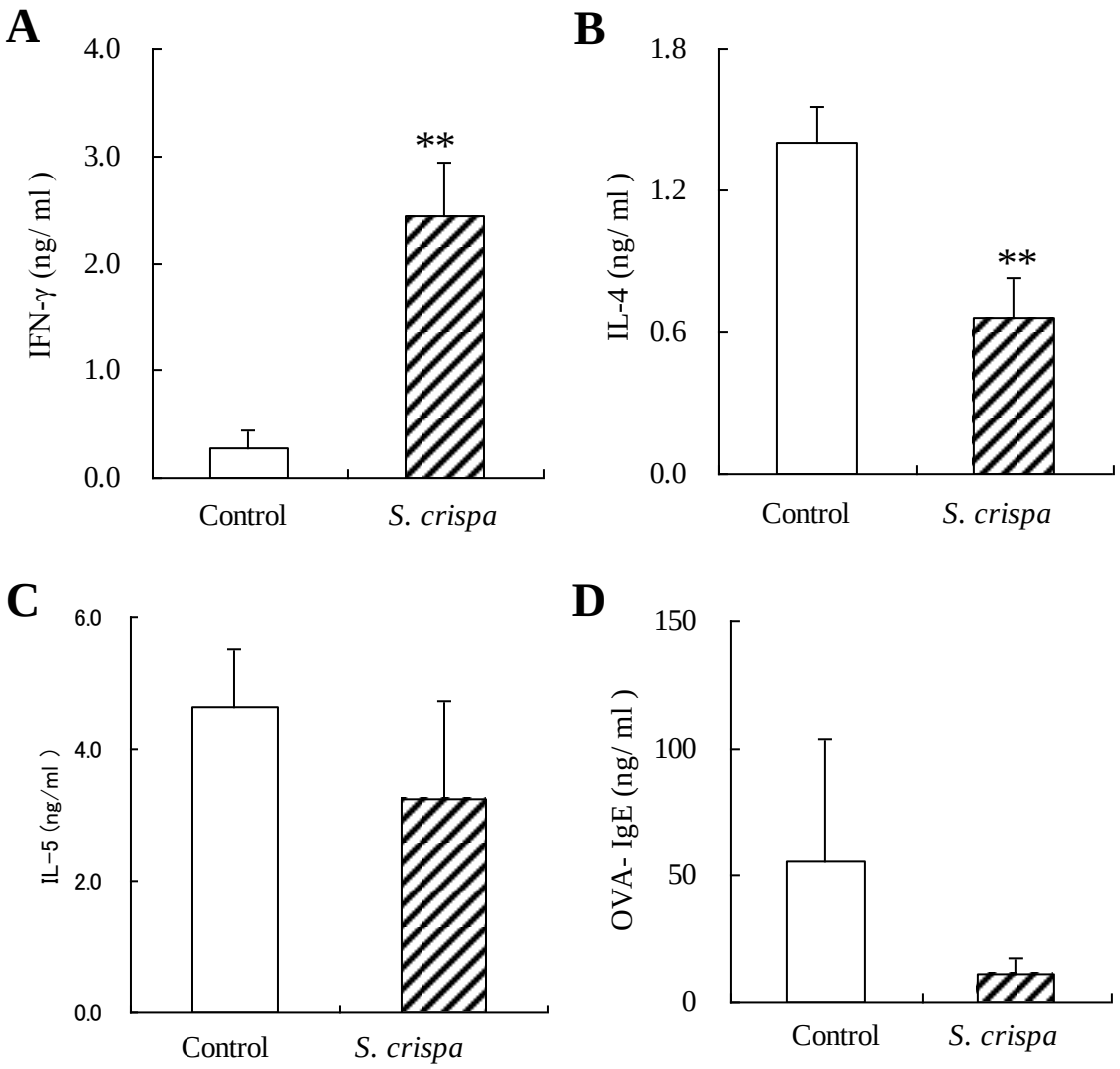

Figure 1. Effects of oral administration of *S. crispa* on IFN-γ, IL-4, IL-5, and OVA-specific IgE secreted by splenocytes derived from OVA-sensitized BALB/c mice.

IFN-γ (A), IL-4 (B), IL-5 (C), and OVA-specific IgE (D). The averages and error bars representing the standard error were obtained from the data of 8 mice, and Student's *t*-test was used for statistical analysis. Control group (open columns); administered group (hatched columns). There was a significant difference of $^{**}p<0.01$ compared to the control group.

Effect of S. crispa on nasal rubbing and sneezing induced by antigen

Figure 2 shows the effects of repeated oral administration of *S. crispa* on nasal symptoms induced by a local application of the antigen in BALB/c mice. The *S. crispa* at doses of 36 or 120 mg/kg was administered orally every day from day 25 to day 53. Both

sneezing and nasal rubbing movements were observed immediately after topical antigen challenge and lasted for more than 30 min. The number of sneezing and nasal rubbing movements was increased progressively by daily intranasal sensitization. The oral administration of *S. crispa* caused a dose-dependent inhibition of the nasal rubbing movements from day 32 to day 53, and a significant effect was observed at a dose of 120 mg/kg on day 53 (Figure 2A). The number of sneezing was also inhibited significantly by oral administration of SC at a dose of 120 mg/kg on day 46 and day 53 (Figure 2B). The effects of *S. crispa* on OVA-specific serum IgE levels in the allergic rhinitis model are shown in Figure 3. The oral administration of *S. crispa* reduced the level of OVA-specific IgE in sera on day 46 and day 53, but showed no significant difference.

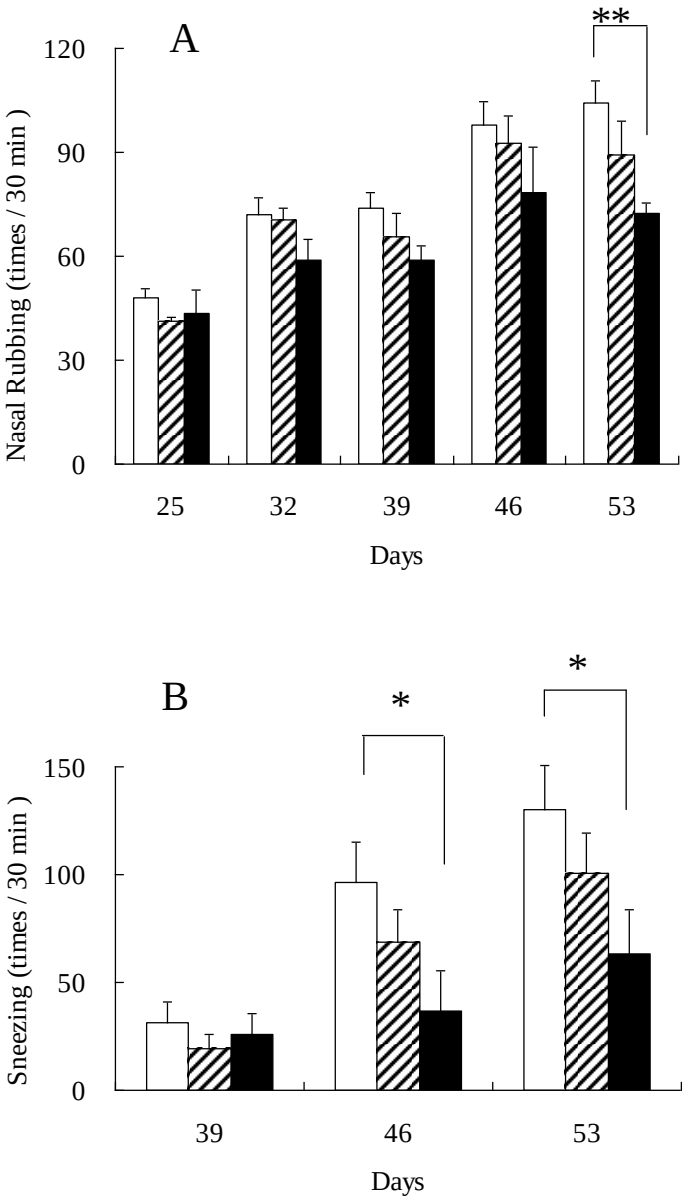

Figure 2. Effects of oral administration of *S. crispa* on symptoms in the allergic rhinitis model.

Mice were repeatedly injected OVA solution (50 μg/10 μl) into the bilateral nostrils every day from day 18 to day 53 after the first immunization and were fed either with or without *S. crispa* daily from day 25. The number of nasal rubbing (A) and sneezing (B) was counted for 30 min after topical application of antigen. The averages and error bars representing the standard error were obtained from the data of 10 mice, and Dunnett's test was used for statistical analysis. Control group (open columns); 36 mg/kg (hatched columns); 120 mg/kg (solid columns). There was a significant difference of $*p<0.05$ and $**p<0.01$ compared to the

control group.

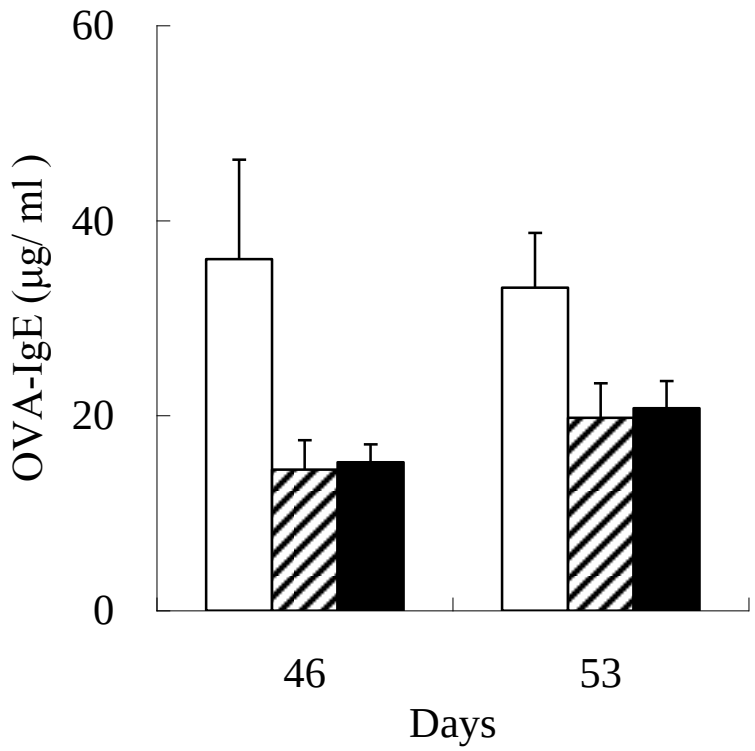

Figure 3. Effect of oral administration of *S. crispa* on OVA-specific serum IgE levels in the allergic rhinitis model.

The averages and error bars representing the standard error were obtained from the data of 10 mice. Control group (open columns); 36 mg/kg (hatched columns); 120 mg/kg (solid columns).

DISCUSSION

It was indicated that oral administration of *S. crispa* could reduce both blood IgE level and scratching number of NC/Nga mice that were induced dermatitis [14]. Moreover, a branched β-glucan from *S. crispa* can induce IFN-γ in DBA/2 mice *in vitro* [15]. Therefore, I investigated whether oral administration of *S. crispa* inhibits IgE production through promoting the Th1-type immune response.

In the present study, oral administration of *S. crispa* inhibited OVA-specific IgE production by OVA-stimulated murine splenocytes through promoting a dominant Th1-type cytokine profile with enhanced IFN-γ and diminished IL-4 and IL-5 production. These results demonstrated that oral administration of *S. crispa* caused inhibition of antigen-specific IgE production through both promoting the Th1-type immune response and inhibiting the Th2-type immune response.

The reduction of the number of sneezing and nasal rubbing movements in the murine allergic rhinitis model seemed to be associated with the reduction in antigen-specific serum IgE levels. In addition, diminished production of IL-4 may be associated with suppression of the symptoms because IL-4 can promote the proliferation of mast cells, which play an important role in allergic reactions as well as allergen-specific IgE antibody production.[19)]

In conclusion, *S. crispa* showed inhibitory effects on immediate allergic reactions, and its mechanism of action is generated mainly by suppression of Th2-type immune response.

In this report, the integrant for immunomodulating activities in *S. crispa* is unknown as the powder of *S. crispa* was used for our experiments. However, there have been some reports that suggest the active ingredient may be β-glucan, which makes up 43.6% of *S. crispa* as measured by the enzyme method [20)]. Thus, *S. crispa* is a good source material for preparing β-glucan in high yields [21)]. Lentinan [22)], schizophyllan [23)], and krestin [24)], all of which contain β-glucan, have been reported to have antitumor activities, and lentinan has been reported to have anti-allergic effects [25)], indicating that β-glucan enhances cellular immunity. Therefore, β-glucan of *S. crispa* may be responsible for anti-rhinitis properties in OVA-sensitized mice.

ABSTRACT

The anti-rhinitis properties of *Sparassis crispa* were investigated in mice. To examine the immunomodulative activity of oral administration of *S. crispa*, splenocytes obtained from ovalbumin-sensitized BALB/c mice fed *S. crispa* were restimulated *in vitro* with the same antigen. Oral administration of *S. crispa* induced IFN-γ, but inhibited IL-4 and IL-5 secretion, and suppressed ovalbumin-specific IgE secretion by ovalbumin-stimulated splenocytes. The effects of *S. crispa* were further investigated by using the allergic rhinitis model in BALB/c mice. Nasal symptoms, sneezing and nasal rubbing induced by ovalbumin challenges were inhibited by oral administration of *S. crispa* in a dose-dependent manner. Furthermore, ovalbumin-specific serum IgE levels were diminished in this model. These results demonstrated that *S. crispa* may be effective in suppressing symptoms of allergic rhinitis through its immunomodulating activities.

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Chapter 6 Dietary *Sparassis crispa* Ameliorates Plasma Levels of Adiponectin and Glucose in Type 2 Diabetic Mice

INTRODUCTION

Research efforts have been increasingly directed towards medical treatment and prevention of metabolic syndrome, such as obesity, type 2 diabetes, and cardiovascular diseases. Type 2 diabetes, a metabolic syndrome disease characterized by hyperglycemia and dyslipidemia resulting from defects in both insulin secretion and insulin sensitivity, has become a significant and growing problem in both developed and developing countries.

It is well known that the adipose tissue is not only a storage depot for fat but also functions as an endocrine organ and plays a key role in the control of endocrine signaling, glucose metabolism, inflammation, and energy homeostasis.[1)] These functions of the adipose tissue are mediated through a number of adipocytokines[1-3)] of which adiponectin has been established as a key adipocytokine in the regulation of metabolic syndrome.[4,5)] Adiponectin also plays a pivotal role in the improvement of insulin sensitivity, as well as in the amelioration of glycemic control and type 2 diabetes.[1,5,6)]

Although there has been considerable progress in the management of diabetes mellitus by synthetic drugs, there is an increasing demand for natural products with antidiabetic activity because of the side effects associated with the use of insulin and synthetic drugs. It has been previously reported that some mushrooms such as *Lyophyllum decastes*,[7)] *Grifola frondosa*,[8)] and *Agaricus blazei*[9)] exhibit antidiabetic activities. However, the effect of mushrooms on glycemic responses and plasma adiponectin levels remains mostly unclear.

Sparassis crispa, known as Hanabiratake in Japanese, is an edible mushroom with various medicinal properties that has recently become cultivable in Japan. *S. crispa* primarily grows on the stumps of coniferous trees and is widespread in northern temperate zones worldwide.[10)] More than 40% of *S. crispa* consists of β-D-glucan, which is comprised of a β-(1,3)-D-glucan backbone with a single β-(1,6) or β-(1,2)-D-glucosyl side-branching unit occurring every three or four residues.[11–13)] *S. crispa* has been reported to have many biological activities such as tumor-suppressing effects,[11,13,14)] improvement in natural killer cell activity,[14)] antiangiogenic effects,[13)] antiallergic effects,[15)] wound healing effects[16)], and enhancements in hematopoietic responses.[11,17)] However, no studies have been performed to elucidate the antidiabetic

properties of *S. crispa*. In this chapter, I examined the beneficial effects of *S. crispa* on glycemic responses and plasma levels of adiponectin and insulin in obese type 2 diabetic mice.

MATERIALS and METHODS

Fungus

The fruit bodies of *S. crispa* cultivated by Unitika Ltd. (Aichi, Japan) were used in the present experiment. These fruit bodies were freeze-dried and ground into an ultrafine powder by a mill. The average diameter of each powder grain was 8 μm.

Animals

Four-week old male KK-Ay mice (Clea Japan, Osaka, Japan) were used in the present study. The mice were housed in an air-conditioned room at 22 ± 2 °C with a 12 h light–dark cycle (light: 7:00 a.m. to 7:00 p.m.). They were kept in an experimental animal room for 7 days with free access to food (CE-2; Clea Japan) and water (tap water) and divided into two groups: 1) the control group, which was fed a diet containing 1.2% cellulose (KC flock; Nippon Paper Chemicals Co., Ltd., Tokyo, Japan), and 2) the SC group, which was fed a diet containing 5.0% *S. crispa*. The total dietary fiber content of each diet was 5.2% and 6.7%, respectively. The mice were fed their assigned diets for 7 weeks. All mice were cared for in accordance with the Guideline for the Care and Use of Laboratory Animals (the Prime Minister's Office No.6, 1980).

Determination of blood glucose, plasma insulin C-peptide, and adiponectin levels

Blood glucose concentrations were monitored once a week during the experimental period. Fasting blood glucose was also measured at 3 and 6 week of experiment using a glucometer (Glucocard G+ meter; Arkray Co. Ltd., Kyoto, Japan). For biochemical examination of Insulin C-peptide II and adiponectin at 3 and 6 week, blood samples were taken from tail vein, and plasma was collected by immediate centrifugation (3000 rpm, 5 min, 4 °C). Insulin C-peptide is released by processing of pro-insulin to matured-insulin and its levels are often measured instead of insulin levels clinically, because it has a longer half-life than insulin. Plasma insulin C-peptide II was measured using a mouse insulin C-peptide II EIA Kit (Yanaihara Institute Inc., Shizuoka, Japan). Adiponectin concentrations were measured using a mouse adiponectin/Acrp30 EIA Kit (R&D systems, Inc., MN, U.S.A.).

Measurement of serum total cholesterol (T-Cho), triglycerides (TG), and cell size of adipose tissue

After receiving their assigned diets for 7 weeks, each mouse was sacrificed by collection of whole blood under pentobarbital anesthesia (50 mg/kg). Serum samples were collected and intra-abdominal adipose tissues (mesenteric, epididymal, and kidney leaf fat tissues) were excised and weighed. Serum T-Cho and TG levels in all mice were determined using the Cholesterol E-test and Triglyceride E-test (Wako, Osaka, Japan). Mesenteric fat tissues of the control and SC groups were stained using hematoxylin-eosin after fixing with 20% neutral buffered formalin (Wako). The area of the adipose tissue cells was measured using image processing software (ImageJ version 1.42q, National Institutes of Health, MD, U.S.A.) under a high-power microscopic field (magnification ×100).

Statistical analysis

All data were represented as mean ± SE. Differences among means were analyzed by the Student's *t*-test or two-way analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

RESULTS

Effects of S. crispa on blood glucose and insulin C-peptide II level, and body weight in KK-Ay mice

The measured blood glucose levels are shown in Figure 1. *S. crispa*-fed KK-Ay mice maintained lower constant blood glucose levels compared to the control mice during the experimental period. Moreover, *S. crispa*-fed KK-Ay mice showed reduced fasting blood glucose and fasting plasma insulin levels, especially significantly at 3 weeks (Figure 2). The temporal changes in body weight are shown in Figure 3. Even after 6 weeks of repeated administration of *S. crispa*, no significant differences in body weight was observed between KK-Ay mice assigned to either the SC or control group.

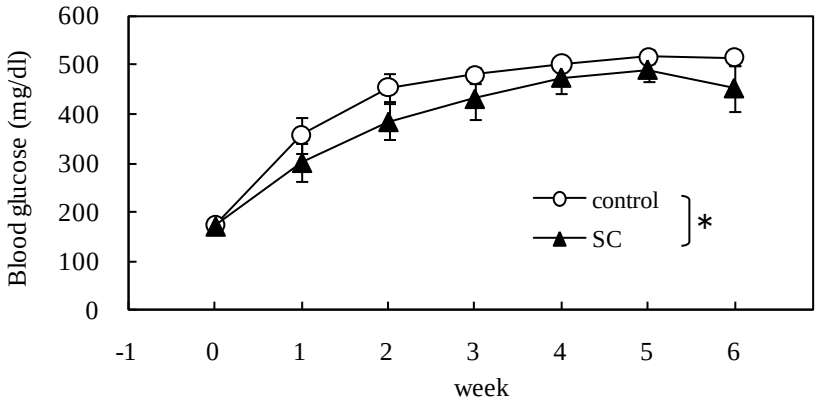

Figure 1. Blood glucose level profiles during the experimental period for KK-Ay mice.

Each value represents the mean ± SE of 6–8 mice. * $P < 0.05$ (vs control group, two-way ANOVA).

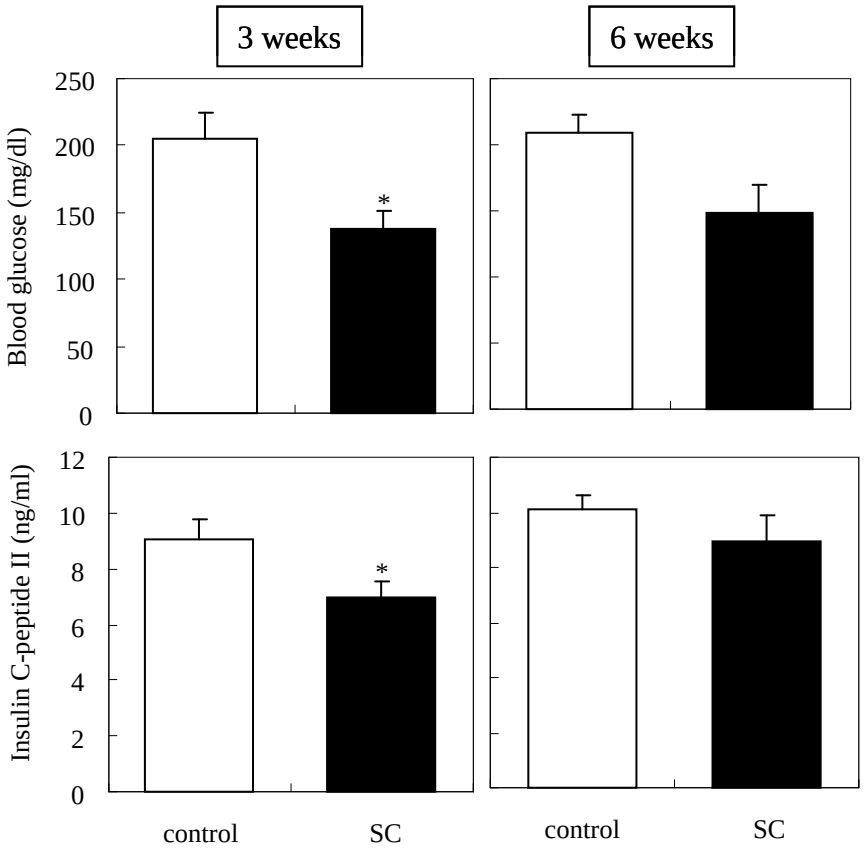

Figure 2. Fasting blood glucose and plasma insulin C-peptide II levels of KK-Ay mice 3 and 6 weeks after administration of the experimental diet. Each value represents the mean ± SE of 6–8 mice. * $P < 0.05$ (vs control group).

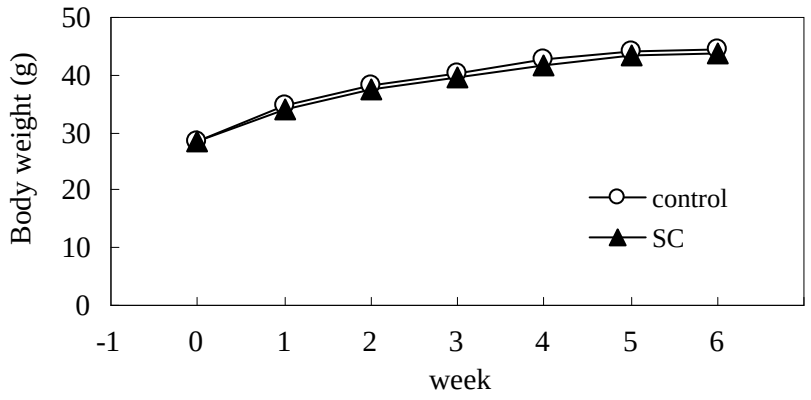

Figure 3. Temporal changes in body weight during the experimental period of KK-Ay mice. Each value represents the mean ± SE of 6–8 mice.

Effects of S. crispa on serum TG and T-Cho levels of KK-Ay mice

The serum TG and T-Cho levels in KK-Ay mice 7 weeks after administration are shown in Figure 4. These levels were relatively lower in the SC group than in the control group.

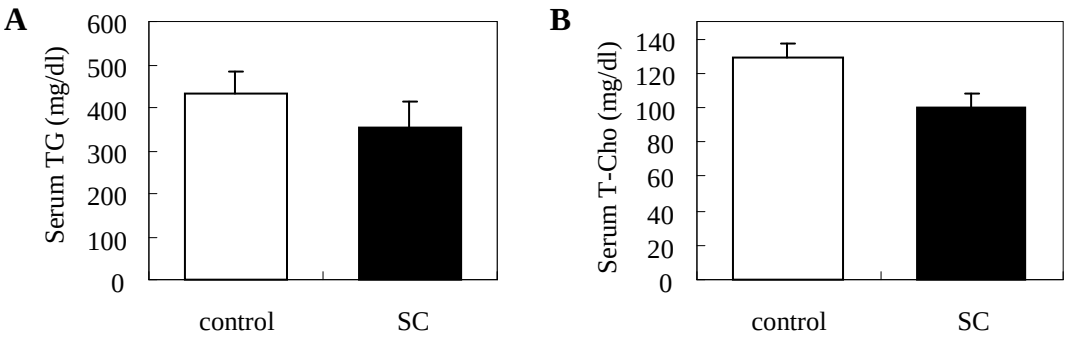

Figure 4. Serum TG (A) and T-Cho (B) levels in KK-Ay mice 7 weeks after administration of the experimental diet. Each value represents the mean ± SE of 6–8 mice.

Effects of S. crispa on plasma adiponectin levels of KK-Ay mice

The plasma adiponectin levels in SC-fed KK-Ay mice 3 and 6 weeks after administration are shown in Figure 5. Plasma adiponectin concentrations were notably higher in the SC group than in the control group.

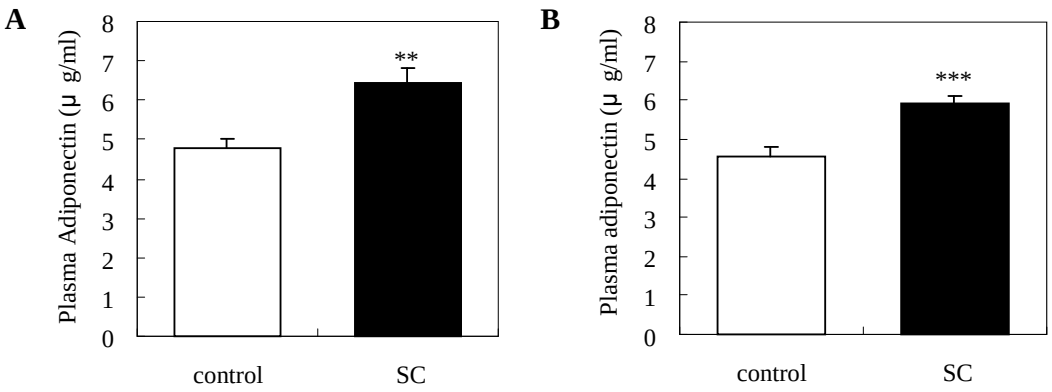

Figure 5. Plasma adiponectin levels in KK-Ay mice (A) 3 weeks and (B) 6 weeks after administration of the experimental diet. Each value represents the mean ± SE of 7–8 mice. ** $P < 0.01$; *** $P < 0.001$ (vs control group).

Tissue weight and cell size of intra-abdominal adipose tissues

The weights and cell size of intra-abdominal adipose tissues are shown in Table 1, and picture of mesenteric abdominal cells representing each group was appeared in Figure 6. Although there were no marked differences in the adipose tissue weight among the dietary groups, the mean of the mesenteric adipose cells size was about 25% smaller in the SC group than in the control group.

Table 1. Weights of intra-abdominal adipose tissues and cell sizes of mesenteric adipose tissue.

group	adipose tissue weight (g)			cell area
	mesenteric	epididymal	kidney leaf	(% vs control)
control	1.13 ± 0.08	1.61 ± 0.04	0.85 ± 0.07	100.0 ± 15.0
SC	1.14 ± 0.06	1.67 ± 0.11	0.90 ± 0.07	75.4 ± 10.3

Each value represents the mean ± S.E. of 4–8 mice.

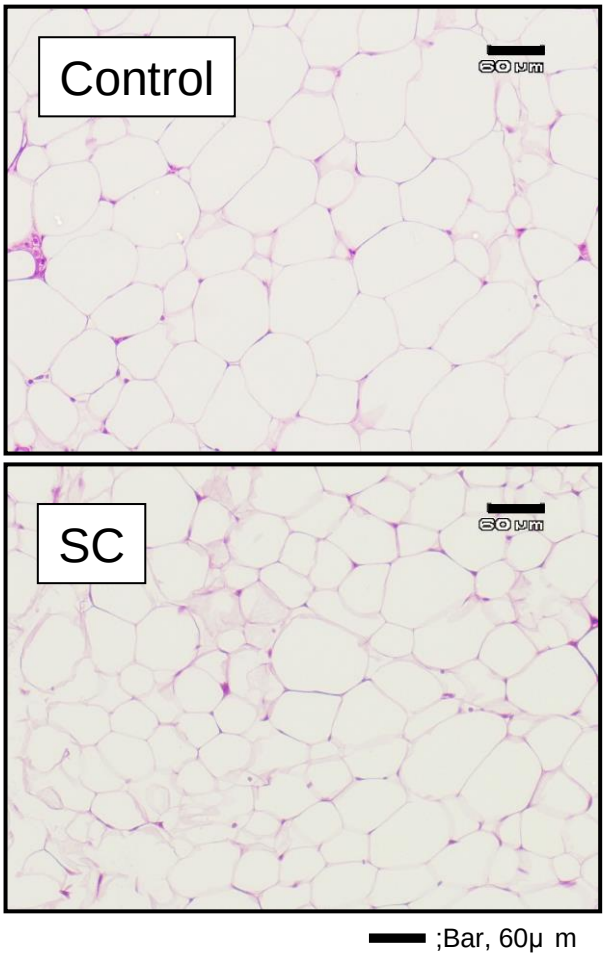

Figure 6. Representative microscope photographs of mesenteric adipose tissues of control group and SC group.

DISCUSSION

In the present study, I observed that the *S. crispa*-fed KK-Ay mice, an animal model of type 2 diabetes mellitus, exhibited significantly increased plasma levels of adiponectin at 3 and 6 week administration of *S. crispa* (Figure 5) as well as a significantly decreased blood glucose and insulin levels at 3 week (Figure 1 and 2) compared to KK-Ay mice fed with a control diet. Moreover, the *S. crispa* diet decreased the serum TG and T-Cho levels, but there were not significant differences (Figure 4). Increase in the level of adiponectin as well as the decrease in the levels of blood glucose and insulin are of particular interest, as there are only a few reports indicating that dietary mushrooms can have beneficial effects on the plasma levels of adiponectin. It is well established that increased adiponectin levels stimulate glucose utilization through the activation of AMP-activated protein kinases in skeletal muscle and liver.[3)] Thus,

administration of *S. crispa* should reduce glucose levels due to improved incorporation of glucose into peripheral tissues in response to *S. crispa*-induced elevated adiponectin levels. Mice lacking adiponectin exhibit severe diet-induced insulin resistance.[18] Furthermore, adiponectin has been found to reverse insulin resistance associated with both lipoatrophy and obesity. Hence, it is suggested that adiponectin is a potent insulin enhancer, linking adipose tissue and whole-body glucose metabolism.[19] The findings of this study strongly support the possibility that dietary *S. crispa* could enhance insulin action through its effect on adiponectin, and suggest that *S. crispa* has the potential to ameliorate or attenuate insulin resistance in type 2 diabetes accompanied by hyperlipidemia.

The size of adipocytes influences the gene expression and secretions of adipocytokines such as adiponectin and tumor necrosis factor-α (TNF–α).[20] No effects on body and adipose tissue weights were observed in *S. crispa*-fed KK-Ay mice (Figure 3 and Table 1). However, the size of the mesenteric adipose cells was relatively smaller in the *S. crispa* group than in the control group (Table 1 and Figure 6). Therefore, it is likely that *S. crispa* feeding might decrease the adipose cell size in response to increased plasma adiponectin levels.

On the contrary, it is well established that treatment with thiazolidinediones or insulin-sensitizing agents increases adiponectin levels through increased peroxisome proliferator-activated receptor-γ (PPAR-γ) activity.[21,22] Therefore, future studies should address the question of whether *S. crispa* feeding modulates PPAR-γ activity.

An epidemiological study has reported an interesting observation that a diet low in glycemic load and high in fiber, obtained from dietary cereal, increases adiponectin levels in diabetic men.[23] Thus, it is likely that a specific kind of fiber in *S. crispa*, such as β-(1,3)-D-glucan, contributed to the observed increase in adiponectin levels. It is very interesting that the antidiabetic effects of this fiber in *S. crispa* are higher than that of cellulose.

In conclusion, this study clarifies the beneficial effects of dietary *S. crispa* on the levels of adiponectin, glucose and insulin. The toxicity of *S. crispa* seems to be very low (LD_{50} >5000 mg/kg body weight). Moreover, *S. crispa*-treated (5000 mg/kg) mice did not show any obviously harmful action (data not shown). Considering the physiological significance of adiponectin in type 2 diabetes, insulin resistance, and cardiovascular disease, the present findings suggest that dietary *S. crispa* has the potential to safely ameliorate these diseases.

ABSTRACT

Sparassis crispa, known as Hanabiratake in Japanese, is an edible mushroom with medicinal properties; however, its antidiabetic activity is not well established. In the present study, I examined the effects of dietary *S. crispa* on diabetic mice. KK-Ay mice that were fed SC for 3 or 6 weeks showed pronounced increase of plasma levels of adiponectin. Significant decrease of blood glucose and insulin levels was also observed by 3 week-administration of *S. crispa*. Moreover, mice that were fed the *S. crispa* diet exhibited relatively decreased serum levels of triglycerides and total cholesterol. Although the *S. crispa* diet had no effect on body and adipose tissue weights in KK-Ay mice, the size of the mesenteric adipose cells of *S. crispa* group was smaller than control group. Thus, the *S. crispa* diet might decrease the adipose cell size in order to increase plasma adiponectin levels. Considering the physiological significance of adiponectin in type 2 diabetes, insulin resistance, and cardiovascular diseases, these findings imply that dietary *S. crispa* has the potential to ameliorate these diseases**.**

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Chapter 7 *Sparassis crispa* Ameliorates Skin Conditions in Rats and Humans

INTRODUCTION

Sparassis crispa, also known as cauliflower mushroom in English and Hanabiratake in Japanese, is an edible mushroom with various medicinal properties that has recently been cultivated in Japan. It is a brown root fungus that grows primarily on the stumps of coniferous trees. It is widely distributed throughout the northern temperate zone. More than 40% of dried *S. crispa* consists of β-glucan, which is composed of a β-(1,3)-D-glucan backbone with a single β-(1,6)-D-glucosyl side-branching unit occurring every three residues.[1)] SC has been reported to exhibit many biological activities, including tumor suppression,[2-4)] cancer prevention,[5)] enhancement of natural killer-cell activity,[3)] anti-angiogenic effects,[4)] anti-allergic effects,[6)] anti-diabetic effects,[7)] platelet anti-aggregation,[8)] HIV-1 reverse transcriptase inhibition,[9)] anti-hypertensive effects,[10)] and enhancement of hematopoietic responses.[11)] Moreover, oral administration of *S. crispa* has been found to promote excisional wound healing in streptozotocin-induced diabetic rodents.[12,13)] However, whether dietary supplementation with *S. crispa* improves skin conditions in animals and humans remains unclear.

In this chapter, to evaluate the effects of *S. crispa* on turnover of the stratum corneum and soluble collagen biosynthesis in dermis, I used collagen synthetic activity-reduced model rats (RMRs) fed a low-protein diet. Collagen synthetic activity in this animal model has been reported to be at the level observed in senescent rats.[14)]

MATERIALS and METHODS

Preparation of collagen synthetic activity-reduced model rats (RMRs)

The composition of the experimental diet is shown in Table 1. All of the samples were prepared by Clea Japan (Tokyo). All animal procedures were performed following the ethical guidelines prescribed by the Animal Study Committee of Unitika Ltd. and the "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain" (Notice no. 88, Ministry of the Environment, Government of Japan). Collagen synthesis was decreased by feeding of the animals with protein-deficient food, but this effect was more pronounced in younger animals.[14)]

Table 1. Composition of the Experimental Diet (%)

Ingredient	6% Protein diet
Milk casein[a]	7.0
Cornstarch	63.0
Granulated sugar	10.0
Corn oil	6.0
Cellulose poIr	5.0
α-Starch	1.0
Vitamin mix[b]	1.0
Mineral mix[c]	7.0

a 85% purity

b Diet #B10000, Clea Japan

c Diet #C10000, Clea Japan

Effects of S. crispa on skin conditions of RMRs

In this study, 5-week-old male Wistar rats (CLEA Japan, Tokyo) were housed in standard stainless steel cages in an air-conditioned room (room temperature, 22°C ± 1°C; humidity, 55% ± 5%) under a 12-h light/12-h dark cycle and were fed a 6% protein diet for 3 weeks *ad libitum*. After treatment, 8-week-old RMRs were obtained. Nine 8-week-old RMRs were divided into three groups. The control group was fed a 6% protein diet; the 70 mg group received oral administration of 70 mg of dried *S. crispa* per kilogram of body weight per day in addition to the 6% protein diet; and the 210 mg group received oral administration of 210 mg of dried *S. crispa* per kilogram of body weight per day in addition to the 6% protein diet. After 3 weeks of feeding, each RMR was administered 5 μL of 5% dansyl chloride (1-dimethylaminonaphthalene-5-sulphonyl chloride) ethanol solution on its back. The rate of desquamation was measured by dansyl chloride fluorescence,[15)] which was visually monitored with a UV lamp (UVGL-58, Funakoshi, Tokyo). The day when fluorescence completely disappeared was defined as the renewal time of the stratum corneum. After an additional 2 weeks of feeding, all of the animals (13 weeks old) were sacrificed. The fur was shaved from the dorsum with electric clippers appropriate for use on small animals, and a 20-cm^2 piece of skin was obtained, weighed, frozen in liquid nitrogen, and immediately stored at -80°C until further analysis. Neutral salt-soluble collagen (tropocollagen) in the dermal skin was extracted by homogenizing the skin in 0.14 M NaCl at 4°C with a homogenizer (PT-10-35 GT, Kinematica, Luzern, Switzerland),

shaking for 20 h at 4°C, and then centrifuging the sample (10,000 rpm for 10 min), as previously described.[16] The supernatant solutions were dialyzed against distilled water and then lyophilized and hydrolyzed with 6 N HCl at 110°C for 24 h. L-Hydroxyproline (Hyp) is an amino acid that exists almost exclusively as a constituent of vertebrate collagen. The content of collagen may be deduced from the amount of Hyp. The Hyp content in the lyophilized residues was determined with an amino acid analyzer (Shimadzu, Kyoto, Japan). The amount of collagen in the skin was expressed as the Hyp content in 20 cm^2 of skin.

Effects of S. crispa on skin conditions of humans

To determine the effects of oral intake of *S. crispa* in humans, I conducted a small-scale randomized, double-blind, placebo-controlled study that included 26 healthy volunteers in Japan, aged 20–60 years. The protocol of the study was approved by the Ethics Review Board of Unitika, and the study was performed in accordance with the Helsinki Declaration. Written informed consent was obtained from all participants. The subjects were divided into 2 groups: *S. crispa*-supplemented (n = 13) and placebo (n = 13). The material for oral supplementation contained 160 mg of *S. crispa* dry powder and olive oil in the form of soft gel capsules. Identical placebo control capsules were prepared with lactose and olive oil in soft capsules. One capsule was orally administered to each subject twice daily. The testing period lasted for 4 weeks, and it began in February 2004. Measurements of transepidermal water loss (TEWL) were done at the beginning of the study and after 2 and 4 weeks. Measurements were done 30 min after the subjects were allowed to rest in a seated position in an environmental test room maintained at 20°C at 60% relative humidity. The TEWL of the left cheek was measured using Tewameter TM300 (Integral, Tokyo).

RESULTS and DISCUSSION

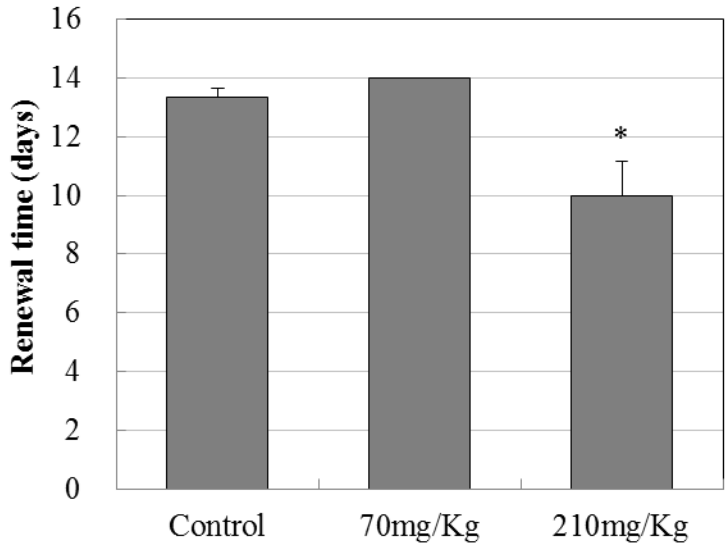
Effects of S. crispa on skin conditions of RMRs

The stratum corneum of the skin functions as a barrier protecting the organism against exogenous substances and preventing water loss. Since it was first introduced by Jansen *et al*.,[15] the dansyl chloride labeling technique has become widely used as a method of measuring stratum corneum renewal time. The principle is simple and depends on fluorochrome labeling of the full thickness of the stratum corneum. The presence of fluorochrome is readily detected under ultraviolet illumination, and the duration it takes the fluorescence to disappear (renewal time) is assumed to be equal to

the time it takes for the entire thickness of the stratum corneum to be exfoliated and replaced with new unstained cells from the dividing epidermis. The animals treated with 210 mg of *S. crispa* (210 mg/kg) demonstrated a significant reduction in renewal time as compared to the control group (Figure 1A).

The amount of collagen in the skin was expressed as the Hyp content in 20cm^2 of skin. The soluble collagen levels for the animals treated with 70 mg (70 mg/kg) and the 210 mg (210 mg/kg) of *S. crispa* were 1.4 and 1.3 times higher respectively than that of the control group (Figure 1B). This indicates that the levels of the newly synthesized collagen, which was hydrophilic and soluble, had increased. Damage, such as that caused by natural aging and photoaging, causes cross-linking of collagen molecules, converting collagen into an insoluble form. This change in solubility contributes to the decrease in water content of the skin with age.[17)]

A.



B.

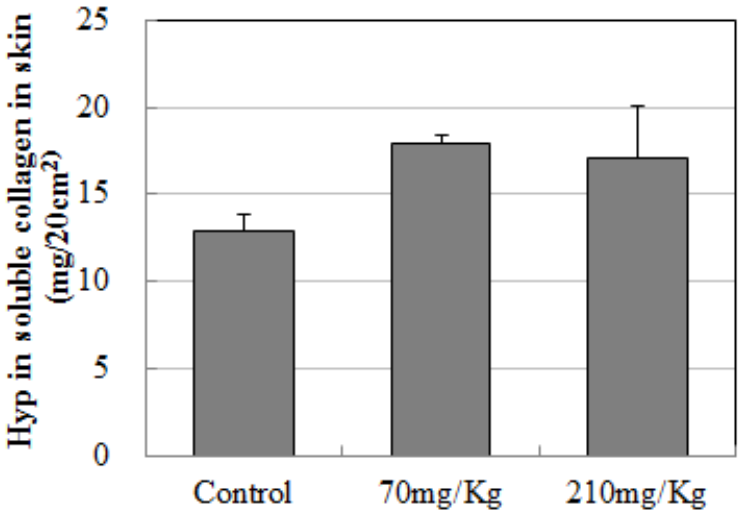


Figure 1. Effects of Oral Administration of *S. crispa* (70 or 210 mg/kg) on the Stratum Corneum and Dermal Layer in RMRs.

A, Effects of the oral administration of *S. crispa* (70 or 210 mg/kg) on stratum corneum renewal in RMRs. B, Soluble collagen content in the skin of RMRs after oral administration of *S. crispa* (70 or 210 mg/kg) for 5 weeks. Values represent mean ± SEM (n = 3). Statistical analysis was done by Dunnet's test. The value marked by an

asterisk was significantly different from that of control at $p < 0.05$.

These results for the RMRs suggest that oral administration of *S. crispa* exhibited positive effects on both the stratum corneum and dermal layer. The renewal time and Hyp content of the animals fed the normal diet (20% protein) were approximately 7 days and 45 mg in 20 cm^2 of skin (data not shown).

Effects of S. crispa on skin conditions of humans

Oral administration of *S. crispa* (320 mg/day) for 28 consecutive days dramatically reduced transepidermal water loss, an indicator of the integrity of the skin barrier. In contrast, the placebo group showed no changes during the testing period (Figure 2). Thus oral administration of *S. crispa* had a positive effect on the skin barrier in humans.

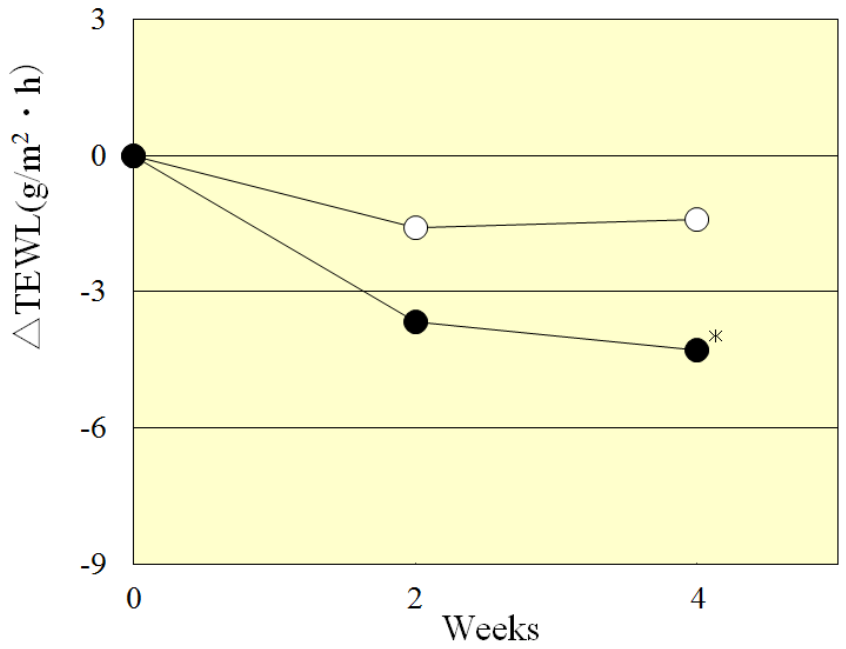


Figure 2. Effects of *S. crispa* -Containing Capsules on TEWL in Healthy Subjects.

TEWL obtained from the cheeks was measured with an evaporimeter at 0, 2, and 4 weeks during the ingestion period. The variation in TEWL relative to the baseline level (ΔTEWL) is shown. ○ indicates placebo, and ● indicates test product. Data are expressed as mean. Statistical analysis was done by Student's *t*-test. The value marked by an asterisk was significantly different from that of placebo at $p < 0.05$.

It has been reported that oral administration of purified β-glucan from *S. crispa* has a suppressive effect on tumor growth and metastasis.[4] In addition, recently I found that wound-healing activity occurred when purified β-glucan from *S. crispa* was administered topically to wounds of diabetic mice.[13] I conclude that β-glucan of this mushroom probably contributes to these improvements in skin condition. But further studies are needed to identify the active component in *S. crispa* definitely and to provide insight into the mechanism of this behavioral response.

In conclusion, I found positive effects induced by *S. crispa* in the skin of rats with accelerated senescence and in healthy human volunteers. To the best of my knowledge, this is the first report suggesting that mushroom intake has a stimulatory effect on the stratum corneum. Moreover, many people in Japan have consumed *S. crispa*, and to date no adverse events have been reported. Thus, *S. crispa* appears to be effective and safe for the improvement of skin conditions.

ABSTRACT

Sparassis crispa is an edible mushroom with various medicinal properties. In this study, I investigated to determine whether *S. crispa* would affect skin conditions in rats and humans. Oral administration of *S. crispa* increased both turnover of the stratum corneum and dermal soluble collagen content in collagen synthetic activity-reduced model rats. To investigate the effects of oral intake of *S. crispa* in humans, I performed a randomized, double-blind, placebo-controlled study. I found that cheek transepidermal water loss was significantly lower in the experimental group than in the control group at 4 weeks of ingestion. This study suggests that *S. crispa* is effective and safe for the improvement of skin conditions.

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General Conclusion

In this study, I have described *S. crispa* as a mushroom species with a great potential for therapeutic applications. The medicinal values of this mushroom were mainly attributable to its abundant 6-branched β-1,3-D-glucan (SBG) contained in the fruit body.

The consecutive ingestion of *S. crispa* fruit body powder significantly suppressed tumor growth, while no such activity was observed on ingestion of the mycelial powder. As the structure of the β-glucan obtained from the mycelia differed markedly from that of the β-glucan obtained from the fruit body, this intergroup difference in antitumor activity might be attributable to the structure and content of β-glucan (Chapter 1).

Dectin-1 and TLR4 have been proposed as SBG receptors. Moreover, treatment with an antibody against dectin-1[1,2] or genetic deletion of TLR4[3] completely prevents SBG-induced maturation of dendritic cells (DCs). These observations indicate that one signaling pathway did not compensate for the other in SBG-treated DCs, which suggests that both receptors are required for SBG action. However, further analysis of the role of these receptors in response to SBG is required to clarify the detailed mechanism of action of SBG in DCs[4].

To date, few studies have described the differences in responsiveness to β-glucans in humans. The studies of differences in reactivity to SBG in different animal strains[5-7] are important from this viewpoint. Further studies on reactivity to SBG could provide clues for developing more effective cancer immunotherapies using SBG.

The mechanism underlying the effects of orally administered SBG remains to be clarified. The results obtained in this study clearly indicate that dietary SBG has some pharmacological actions. Therefore, it must be assumed that orally ingested SBG interacts with either intestinal epithelial cells and/or intestinal DCs, which ultimately results in the priming or activation of other immune cells.

The antitumor mechanisms of SBG, except for its immunomodulatory actions, have not been sufficiently studied thus far. Thus, I tried to elucidate the possible mechanisms of anti-angiogenic effects of SBG (Chapter 2). The findings showed that SBG had both anti-angiogenic functions and anti-metastatic effects on neoplasm in different animal models.

The antitumor effects of SBG may be partially attributable to its anti-angiogenic actions.

Many studies about the antitumor activity of edible mushrooms have particularly focused on β-D-glucan. However, few studies have focused on antitumor components other than β-D-glucan. In this study, I have demonstrated that *S. crispa* had some low molecular weight constituents with antitumor activity, such as hanabiratakelide (Chapter 4) and FHL (Chapter 3).

Little has been reported on the pharmacological effects of *S. crispa* except for the antitumor effects. I have elucidated the various pharmacological activities of *S. crispa* using different animal models. Dietary *S. crispa* ameliorated allergic rhinitis (Chapter 5), diabetes (Chapter 6), and skin conditions (Chapter 7).

Many people in Japan consume *S. crispa*, and to date, no adverse events due to *S. crispa* consumption have been reported. Therefore, dietary treatment with *S. crispa* may prove to be a safe therapy for cancer and other chronic diseases.

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List of Publications

Chapter 1

Kimura T, Yamoto K, and Nishikawa Y, " Comparison of the antitumor effect of the fruit body and the mycelia of Hanabiratake (*Sparassis crispa*)," *MUSHROOM SCIENCE AND BIOTECHNOLOGY*, in press (2013).

Chapter 2

Yamamoto K, Kimura T, Sugitachi A, and Matsuura N, "Anti-angiogenic and anti-metastatic effects of beta-1,3-D-glucan purified from Hanabiratake, *Sparassis crispa*," *Biol. Pharm. Bull.*, **32**, 259-263 (2009).

Chapter 3

Yamamoto K, Nishikawa Y, Kimura T, Dombo M, Matsuura N, and Sugitachi A, "Antitumor activities of low molecular weight fraction derived from the cultured fruit body of *Sparassis crispa* in tumor-bearing mice," *Nippon Shokuhin Kagaku Kogaku Kaishi*, **54**, 419-423 (2007).

Chapter 4

Yoshikawa K, Kokudo N, Hashimoto T, Yamamoto K, Inose T, and Kimura T, "Novel phthalide compounds from *Sparassis crispa* (Hanabiratake), Hanabiratakelide A-C, exhibiting anti-cancer related activity," *Biol. Pharm. Bull.*, **33**, 1355-1359 (2010).

Chapter 5

Yao M, Yamamoto K, Kimura T, and Dombo M, "Effects of Hanabiratake (*Sparassis crispa*) on allergic rhinitis in OVA-sensitized mice," *Food Sci. Technol. Res.*, **14**, 589-594 (2008).

Chapter 6

Yamamoto K and Kimura T, "Dietary *Sparassis crispa* (Hanabiratake) ameliorates plasma levels of adiponectin and glucose in type 2 diabetic mice," *J. Health Sci.*, **56**, 541-546 (2010).

Chapter 7

Kimura T, Hashimoto M, Yamada M, and Nishikawa Y, "*Sparassis crispa* (Hanabiratake) Ameliorates Skin Conditions in Rats and Humans," *Biosci. Biotechnol. Biochem.*, in press (2013).

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